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XCVIII. THE DIETETIC VALUE OF BARLEY, MALT AND MALTED LIQUORS AS DETERMINED BY THEIR VITAMIN CONTENT.

By HERBERT WILLIAM SOUTHGATE.

(*Pharmacological Department, University of Sheffield.*)

(*Received May 7th, 1924.*)

The investigation of malted beverages and their precursors is not new. Harden and Zilva [1918] found bottled ale and stout, as well as beer, to be lacking both in the antineuritic and antiscorbutic factors. These authors considered the water-soluble B factor and the antineuritic factor to be identical, and used pigeons to test for this vitamin, while guinea-pigs and monkeys were employed in the tests for the presence of the antiscorbutic factor. They also found that 40 g. of malt when fed alone to pigeons did not prevent or even delay the onset of symptoms of polyneuritis in the birds. Some earlier work by Cooper [1912, 1914] on the antineuritic properties of the precursors of beer, should be mentioned here. This author [1912] found that a diet containing 13 g. of polished rice, 2 g. of raw beef, and 5 g. of barley (unhusked, 12 % water content) would recuperate pigeons in the early stages of polyneuritis resulting from diets consisting too exclusively of polished rice. Also [1914] as a result of testing out three different samples of malt extract for their efficiency in curing polyneuritis in pigeons, he found that two of the samples had a rapid curative action, whereas the third even in large doses had no effect. Steenbock, Kent and Gross [1918] having experimentally examined the dietary qualities of barley with regard to its effects on the growth of rats, concluded that this cereal contained an abundance of water-soluble B vitamin. When fed on a ration containing barley 40, butter-fat 5, salts 3·7, caseinogen 18, and dextrin 33·3, rats showed entirely normal growth curves, whilst when the barley content was reduced to 20 % growth was not quite normal. With regard to the fat-soluble A vitamin content, however, they reported "The amount of fat-soluble vitamin introduced with 15 % of barley in the ration, so far as improvement in growth is concerned, is negligible, 60 % however improves the ration markedly."

In the course of the present investigation barley, malt (kilned), unfined and clear ale have been systematically tested for the presence of accessory food factors with the results recorded below.

Fat-soluble A vitamin was tested for in the usual way with rats. The experience of previous workers with cereals and the effect of heat on these

suggested a negative result, except perhaps in the case of the barley. This was borne out by experimental results, and even in the case of the original barley a diet containing 50 % unhusked barley and representing about 7 g. of barley per rat per day failed to produce normal growth. An equal ration of malt in the diet also failed to prevent the onset of symptoms due to lack of vitamin A in the diet or to bring about any amelioration of the symptoms when these had developed on a vitamin A deficient diet. Both fined and unfined beers were tested for the presence of this vitamin. It was calculated that 20–30 cc. of these liquids respectively were consumed per rat per day, given by mixing the liquid with the dry food, or by filling the animals' drinking flasks with it. Some fined beer was also evaporated down under reduced pressure at 60° C. to a quarter the original volume and 20–30 cc. of this were given to rats, but in all cases, whether tested to prevent the onset of symptoms or given in the attempt to cure, the results were negative.

The presence of the antiscorbutic factor was tested for on guinea-pigs with the usual basal diet of oats and bran to which barley, malt, fined or unfined beers were added. A ration containing 60 % of barley or malt neither protected nor cured; and the result which Harden and Zilva [1918] obtained when testing for the antiscorbutic factor in pale ale and malt on guinea-pigs was confirmed by similar experiments in the present series. The residues in 5 g. lots, obtained by evaporating down fined and unfined samples of beer under reduced pressure neither protected nor acted curatively.

The testing for the presence of water-soluble B vitamin in the substances under investigation was carried out on rats of weights 30–35 g. at the commencement of the experiments, the temperature of the rat room being maintained at 21° C. as nearly as possible. Samples of English Standwell, English Chevalier, Californian, Chilian, Czecho-Slovakian and Australian malt, together with some of their original corresponding barley samples were obtained. The former were procured directly after malting and bottled in fruit-jars, the barley samples were also kept bottled. The water content of the malt averaged 3 % and that of the barley about 14 %, so that in the feeding experiments 11 g. of barley were considered equivalent to 10 g. of malt. It should be mentioned here that the malt samples used in this investigation were treated by the following kiln-drying process:

24 hours'	drying	to	90° F.
24 ,,	,,	,,	130° F.
24 ,,	,,	,,	180° F.
6–8 hours' final curing at			200° F.

Experiments were first directed to ascertain the minimum amount of each of these substances required to be added to the basal ration to give a normal growth curve for the rats on a basal diet of 24 % caseinogen, 47·5 % starch, 14·5 % cane sugar, 10 % purified butter-fat, 4 % dry salt mixture. Rats having 0·5 g. of malt added to their diet or the corresponding weight of barley did not show normal growth (Fig. 1). Those having 1 g. of malt or 1·1 g. of

barley (which at the beginning of the feeding represented about 30 % of the total ration) showed normal growth curves (Fig. 2), but no pregnancies. When the amount given daily was 1·5 and 1·7 g. respectively there were good growth curves with pregnancies. Fig. 3 shows the average growth curves up till pregnancy for the six samples (English Standwell, Chevalier, Chilian, Californian,

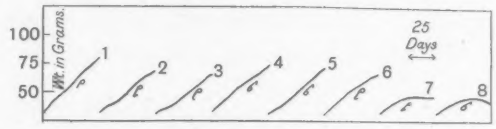

Fig. 1. Curves 1–3 show the effect of adding 0·5 g. of malt to the rats' basal diet. Curves 4–6 the effect of adding 0·5 g. of barley to the basal diet. Curves 7 and 8 for rats on basal diet only.

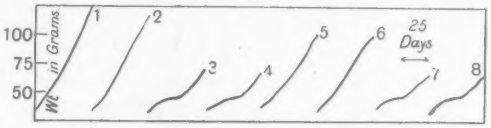

Fig. 2. Curves 1 and 2 the effect of adding 1 g. malt to basal diet protectively. Curves 3 and 4 the same quantity of malt added when growth had ceased on basal diet. Curves 5 and 6 1·1 g. barley added to basal diet protectively. Curves 7 and 8 the same amount of barley added to the basal diet when growth had ceased on this, indicated by arrowhead.

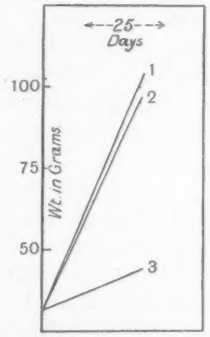

Fig. 3. Curve 1 is the average growth curve of twelve rats, pairs of which had 29 % of the six samples of malt respectively in their dry ration. Curve 2 is the average growth curve of the twelve rats, pairs of which had 29 % of the corresponding six samples of barley respectively in their dry food ration. Curve 3 is the average growth curve for four rats on the basal diet only.

Australian, Czecho-Slovakian) of malt and their corresponding barleys, fed protectively to six pairs of rats along with two pairs of controls on basal diet only. In this case the rats were drawn from four families. The malt or barley in this experiment always formed a definite proportion (29 %) of the dry ration. These curves have been averaged to save space since the result in each case was the same—the rats on malt showing a slightly better growth curve than those on the corresponding barley. It seemed possible at first that these

results indicated a definite synthesis of water-soluble B vitamin on germination of barley, just as the antiscorbutic vitamin is formed under these conditions. However, the difference is so small that a more probable explanation for the small difference would seem to be in the increased ease of assimilation of malt due to the fact that the process of malting increases the solubility of the total solids of the barley kernel.

These results, therefore, show that the water-soluble B vitamin content of barley as determined by its influence on the growth of rats is not affected by the process of malting.

As already stated, Harden and Zilva [1918] assuming the identity of water-soluble B vitamin with the antineuritic factor found in the case of one sample of malt that 40 g. of this did not even delay the onset of polyneuritis in pigeons, whereas Cooper [1914] found that of three samples of malt extract two of them in quantities of 5 and 7 g. respectively and therefore corresponding to about 6 and 8 g. of actual malt cured pigeons with polyneuritis following an exclusive diet of polished rice, whereas the third sample in 10 g. doses

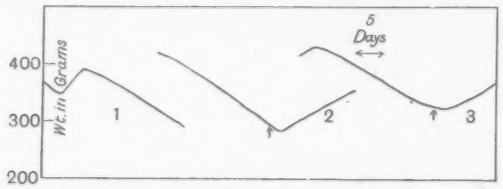

Fig. 4. Curve 1 shows the growth curve of pigeon *A*. Curve 2 that for pigeon *B*. Curve 3 the growth curve for pigeon *C*.

(corresponding to about 11 g. of malt) had no curative effect. In the present investigation some of the malt from the English Chevalier sample of barley which brought about growth in rats in 1·5 g. doses daily was tested curatively on three pigeons. The birds were fed on the same basic diet as was used in the rat experiment till they showed early symptoms of polyneuritis and then malt was added to their diet. At first they were fed naturally with the dry mixture, made up into pellets with about an equal weight of water. At the commencement of the experiment it was judged that about 30 g. of the moist basal food was eaten daily per bird, but as the experiment progressed loss of weight and corresponding decline in appetite had to be recorded. Pigeon *A* (Fig. 4) after 24 days developed very marked symptoms of polyneuritis during the night and died next day. Pigeon *B* (Fig. 4) on the 20th day showed twitchings of its wings and was forcibly fed with 10 g. of malt and 5 g. of dry basal food daily. Pigeon *C* (Fig. 4) showed retraction of the neck on the 24th day and was then fed forcibly a similar malt ration to *B*. The symptoms showed rapid improvement while the weights in both cases improved also. Thus the same sample of malt which when added to the basal diet of rats gave normal growth was found to have curative effects when added in 10 g. doses (the minimum was not determined in this case) to the diet of pigeons, in which

symptoms of polyneuritis had been developed through feeding with the same basal diet as the rats were having. The negative result which Harden and Zilva obtained with 40 g. of malt may have been due to variation of the vitamin content with sample—one of the malt extract samples tested by Cooper [1914] did not cure in 10 g. lots. The experience in the present investigation has however been that the vitamin B content of malt as determined with rats has been constant for the eleven different samples tested. Nor does it seem that the reason for the conflicting results should be looked for in the possible non-identity of the water soluble B and antineuritic factors. Funk [1914] on feeding pigeons with the same balanced basal diet as rats were having produced symptoms of peripheral neuritis both in the birds and animals, a result which has been corroborated in the present investigations. Levene and Muhlfeld [1923] have sought to revive the controversy as to the identity or non-identity of the above-mentioned factors, but their claim "to have obtained additional evidence in support of the view that the antineuritic and growth-promoting principles are not identical" would seem doubtful on the simple ground of their experimental results, for the two samples of yeast which gave the "less satisfactory" results with rats are the two (of the four under test) which did not give good results when tested on pigeons. But a more serious objection to their claim seems to lie in the planning of their experiments, for while they are careful to give their rats a well-balanced basal diet they are content to feed their pigeons artificially with polished rice which gives an ash of about 0·5 % (calcium content very small) and fat about 0·25 %. The paper of Steenbock and his collaborators [1918] to which reference has already been made contains several interesting charts which illustrate the need for balancing the diet of rats. These authors showed that barley alone fed to these animals would not give normal growth, that barley fortified with fat-soluble vitamin improves the growth curves, that salt additions improve barley as a food more than the addition of either fat-soluble vitamin or protein and so on. E. Mellanby [1921, 1922] as a result of his work on rickets insists on the need for a balanced diet when testing out a particular ingredient. His results led him to the conclusion that "the minimum of any substance essential for perfect health and development is a variable, dependent on the other factors of the diet eaten." His later work on cereals [1924] further led him to the conclusion that there is in cereals a positive toxic factor tending to produce rickets, and that a larger amount of the antirachitic vitamin is therefore required to neutralise the added effect of this agent. Thus, as a result of feeding oatmeal, barley, rice, rye and white flour respectively in equal amounts, other constituents being the same, to a litter of puppies, he found that oatmeal produced by far the worst rickets and white flour least—a result not to be explained by the difference in inorganic salts content or carbohydrate. The worst rickets could be prevented by the addition to the diet of small quantities of cod liver oil. In the light of these results and those of Steenbock and his co-workers one may well ask whether it is a fair experiment to test for the presence of

one essential ingredient of the diet of an experimental animal or bird whose diet has consisted wholly of barley or of polished rice. It is here that the critical analysis by Mitchell [1919] of the experimental evidence in favour of the identity or non-identity of the water-soluble growth-promoting vitamin with the antineuritic vitamin would seem to lose much of its value; for after stating that "polished rice, however, is defective in several factors beside the antineuritic factor. It is, therefore, probable that foods containing comparable amounts of this vitamin may be unequally effective in preventing or curing the polyneuritic symptoms, depending upon the extent to which they supplement polished rice in these other respects. Conversely foods containing unequal concentrations of the antineuritic principle may have their relative values as sources of this vitamin distorted for the same reason," and then illustrating this by reference to the experience of Campbell and Chick [1919] who found that a ration of 60 cc. of autoclaved milk added to the basal diet of oats, bran and water delayed by one to three weeks the normal onset of scurvy in guinea-pigs, he then proceeds to compare results obtained with unequal unbalanced diets.

In fine therefore it may be urged that it is impossible to determine the food value of any constituent of diet where the animal or bird under experiment is not obtaining a balanced diet, and that where it is desired to compare the effect of a constituent on two species these should be fed with the same balanced diet. Thus it seems probable that the different conclusions that have been arrived at by various investigators both as to the question of the identity of the antineuritic and water-soluble B vitamins and as regards this particular investigation, the question of the presence of vitamin B in barley and malt, would disappear if the basal diets used were strictly comparable in composition. The growth stimulant effect and the curative effects of barley and malt in the above described experiments, when used in conjunction with the same basal diet, suggest the identity of the active agents.

The results of testing watery extracts both fermented and unfermented of malt will now be given and then discussed. Fig. 5 shows the result of giving rats bottled beer of a well-known brand purchased from a local shop. Those being fed protectively were given 10 cc. at the start which was afterwards increased to 17 cc. per day per rat. Those tested for a cure were given 17 cc. from the commencement. The results of giving similar amounts of wort of original gravity 1053 and of the fined beer from this are shown in Fig. 6. Fig. 7 shows the result of feeding 5 g. (wet) of brewer's grains, ground up in an Excelsior mill, replacing 1 g. of the dry basal food. In Fig. 8 are compared the growth curves of rats given 10 cc. increasing to 17 cc. of unfined beer (original gravity 1053) with those of rats on a similar quantity of this unfined beer after it had been centrifuged for 10 minutes at 4000 r.p.m. to remove yeast cells.

These results for the water-soluble B vitamin content of malt, wort and beer show that in the process of brewing some of the vitamin present in the

malt disappears before the fined beer stage is reached. For to take the case of the results illustrated in Fig. 5 an original gravity of about 1055 may be assumed for this beer. This corresponds to 1 g. of malt for every 4·8 cc. of the fined product, so that 17 cc. of this should give an excellent growth curve, whereas only subnormal results were obtained. It is true that in this case the protective figure for the actual malt sample could not be obtained, but this

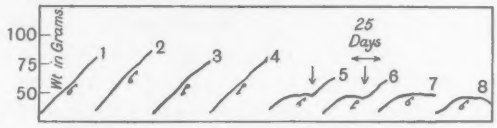

Fig. 5. Curves 1–4, 10 cc. increased to 17 cc. of Bass added daily to the basal diet. Curves 5 and 6 17 cc. of Bass were added daily from the point indicated by the arrowhead. Curves 7 and 8 those for rats on the basal diet only.

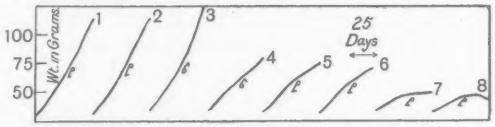

Fig. 6. Curves 1–3 show the effect of giving 10 cc. increasing to 17 cc. of wort (gravity 1053) with the basal diet. Curves 4–6 the effect of giving similar amounts of the fined beer from this wort. Curves 7 and 8 rats on basal diet only.

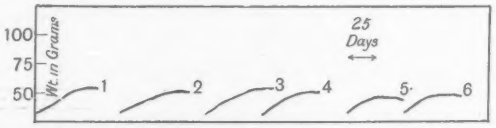

Fig. 7. Curves 1–4, 5 g. (wet) of brewer's grains ground up and replacing 1 g. of the dry basal food. Curves 5 and 6 rats on basal diet only.

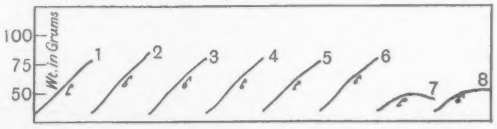

Fig. 8. Curves 1–3, rats had 10 cc. increasing to 17 cc. of unfined beer (original gravity 1053) added to their basal diet. Curves 4–6 rats had a similar quantity of this unfined beer after centrifuging. Curves 7 and 8 rats on basal diet only.

figure (1·5 g.) was found to hold for the malt sample from which the beer was made which was given to the rats whose growth curves are shown in Fig. 8. As the original gravity was 1053, every 17 cc. of the beer corresponded to 3·5 g. of malt, which would give an excellent growth curve if no vitamin were lost. From the results with the brewer's grains it would seem that only small amounts of vitamin are retained at this stage. There is the possibility that some of this factor may be absorbed and carried down during the process of hopping [cf. Drummond, 1917], or that the yeast cell itself during the fermentation process may actually absorb it. (Experiments are being carried

out to test this latter possibility.) The usual process of fining does not appear to be the stage at which the vitamin is lost, for samples of fined ale and centrifuged unfined ale brewed from a wort of original gravity of 1045 and therefore corresponding to 3 g. of malt per 17 cc. of beer show no difference in their effects on the growth of rats. The experimental results therefore show:

(1) That both barley and malt contain water-soluble B, as judged by the effect on rats, in considerable and equal amounts.

(2) Malt was also found to prevent and cure readily polyneuritis in pigeons when used in conjunction with the same basal diet as was used in the experiments on growth in rats. The probable explanation of the discrepancies in the results obtained by different investigators on the subject is discussed, as well as the related subject, the identity of vitamin B with the antineuritic factor.

(3) Beer also contains vitamin B independently of its yeast content but to a much less extent than that in the corresponding amount of malt used in its manufacture.

(4) There is no evidence of the presence to any appreciable extent of the other two accessory food factors in barley or its derivatives.

These experiments have been carried on in the laboratory of Professor Edward Mellanby, to whom I would express my indebtedness for his constant support and helpful criticisms. My thanks are also due to Mr W. T. Wright of the Sheaf Brewery, Sheffield, for kindly supplying material and for advice on the practical side of the malting and brewing industries.

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REFERENCES.

Campbell and Chick (1919). *Lancet*, ii, 320.
Cooper (1912). *J. Hyg.* **12**, 436.
—— (1914). *J. Hyg.* **14**, 12.
Drummond (1917). *Biochem. J.* **11**, 255.
Funk (1914). *Z. physiol. Chem.* **89**, 373.
Harden and Zilva (1918). *J. Inst. Brewing*, **24**, 197.
Levene and Muhlfeld (1923). *J. Biol. Chem.* **57**, 341.
Mellanby (1921). *Medical Research Council. Special Report Series*, No. 61.
—— (1922). *Brit. Med. J.* ii, 4th Nov.
—— (1924). *Proc. Roy. Soc. Med.* **17**, No. 6.
Mitchell (1919). *J. Biol. Chem.* **40**, 399.
Steenbock, Kent and Gross (1918). *J. Biol. Chem.* **35**, 61.

XCIX. ON THE CHOLESTEROL CONTENT OF THE BILE, BLOOD AND FLESH OF THE HIPPOPOTAMUS.

By JOHN ADDYMAN GARDNER.

From the Biochemical Laboratory, St George's Hospital.

(*Report to the Medical Research Council.*)

(*Received May 21st, 1924.*)

Our knowledge of the origin and function of cholesterol in the animal organism, or of the processes involved in its storage and mode of transit to tissues requiring it, is still very scanty in spite of the very large amount of work expended on the subject in recent years.

In 1909 Dorée and Gardner from a consideration of the facts then available were led to formulate the following hypothesis: (1) cholesterol is a constant constituent of all cells, and when these cells are broken down in the life process their cholesterol is not excreted as a waste product, but is utilised in the formation of new cells; (2) a function of the liver is to deal with and break down products of dead cells, *e.g.* blood corpuscles, and to eliminate the cholesterol in the bile; (3) after the bile has been poured into the intestine in the process of digestion, the cholesterol is reabsorbed, possibly in the form of esters, along with the bile salts and carried by the blood to the various centres and tissues for reincorporation into the constitution of new cells.

This hypothesis received considerable support from the experiments of Dorée, Ellis, Fraser, Gardner and Lander [1908–1913], Wacker and Hueck [1913], who succeeded in demonstrating both in the case of herbivora—such as rabbit—and carnivora—such as dog and cat—a connection between the cholesterol ingested with the food and the amount in the blood, and from the careful investigation of Mueller [1915] who showed by analysis of chyle obtained from thoracic duct fistulae in dogs that cholesterol is readily absorbed from the intestinal tract through the chyle and may be recognised in the contents of the thoracic duct. The question whether the excretion and subsequent absorption of the cholesterol of the body forms a regular and exact cycle, or whether there is any wastage of cholesterol which would require to be made up by the animal either by actual synthesis in the body from simpler substances or by the utilisation of that taken in the food or by both processes is at present difficult to answer. The domestic herbivorous animals do not excrete appreciable quantities of cholesterol, or any recognisable derivative

of this substance in their faeces [Dorée and Gardner, 1908, 1], whilst their bile contains considerable quantities, in the case of cow for instance 0·07 %.

Loss however may occur in other ways. In man the sweat and sebum have been shown to contain cholesterol; in sheep the presence of relatively large quantities of cholesterol and cholesteryl esters has been demonstrated in wool fat: considerable quantities are also eliminated in milk.

The food of the herbivora contains no cholesterol, but instead the closely related sterols of the phytosterol group. Evidence has however been brought forward [Fraser and Gardner, 1910] which indicates that phytosterol can be absorbed and appear in the blood as cholesterol, but data are yet wanting to form any balance sheet of intake and output in various ways.

In the case of carnivora—dog and cat—cholesterol is a normal constituent of their food, and feeding experiments [Dorée and Gardner, 1908, 2; Kosumoto, 1908; Ellis and Gardner, 1909, 2] have shown that the total cholesterol found in the faeces can be approximately accounted for by that taken in with the food, provided the animal remains healthy and constant in weight. Nevertheless in experiments in which the intake of cholesterol was reduced to a minimum, *e.g.* on diets of bread, egg white and cream, etc., traces of sterols of the order of 0·03–0·05 g. per day were excreted.

On the other hand, in man the state of affairs is somewhat different. The adult human subject is marked off from other animals by the fact that the cholesterol passed into the intestines undergoes reduction at some stage to coprosterol and β-cholestanol, apparently by bacterial action, though a small quantity of cholesterol escapes this process [Gardner, 1921]. It would seem a probable assumption that this reduction limits the reabsorption of cholesterol, though this has not been definitely proved. Careful experiments on adults [Gardner and Fox, 1921] have shown that there is an average negative balance of 0·3 g. per day, so that there must be some synthesis going on in the body. Similar results have been published by Thannhauser [1923].

In recent years the chemical investigations of Wieland and his co-workers on the bile acids [1912], and of Windaus [1910] on the oxidation of the hydrocarbons, cholestane and coprostane, have definitely proved that cholic acid and coprosterol have the same carbon skeleton and that cholic acid only differs in this respect from the latter by the loss of a $CH(CH_3)_2$-group. Windaus has, we believe, gone so far as to suggest that the bile acids may be derived from the cholesterol in the organism. If this be the case any bile acids lost in the faeces would have to be reckoned with in estimating the output of sterol derivatives, and the above-mentioned negative balance would be considerably increased. The hypothesis is however we think scarcely borne out by the results of Thannhauser. We think it more likely that the two classes are produced by collateral processes from a common origin.

In some of the modern text-books of physiological chemistry it is stated that the bile of the hippopotamus differs from that of other animals in containing no cholesterol. For instance, A. P. Mathews in his text-book of

Physiological Chemistry (1921, p. 432) states that "cholesterol has been found in the bile of all animals in which it has been looked for, with the exception of the hippopotamus," and on p. 434 he again draws attention to this and goes on to suggest "it would be interesting to know whether the blood of the hippopotamus contains cholesterol. Perhaps some African physiologist may make some interesting discoveries by studying the bile and blood of this animal." The statement is based on some work of Hammarsten [1911]. On looking up the original paper we found that the statement in such definite form was perhaps scarcely warranted. Hammarsten found that the bile of the hippopotamus was relatively poor in ether-soluble matter. From the bile of two animals he only obtained 0·756 g. of ether extract. 0·2 g. he used in testing for the presence of phosphorus compounds and the rest he hydrolysed with alcoholic potash in order to examine the unsaponifiable matter. He states "Der Nachweis von Cholesterin gelang jedoch nicht, als Endprodukt wurde nur eine äussert kleine Menge einer durchsichtigen firnisähnlichen Masse erhalten." He does not explain how he treated this unsaponifiable matter, or how he tested for cholesterol. In his general conclusions at the end he remarks "Cholesterin konnte nicht nachgewiesen werden, und die in reinem Äther löslichen Stoffe, deren Menge nur sehr klein war, bestanden hauptsächlich aus Olein mit ein wenig festem Fett." This statement of Hammarsten with regard to the bile of the hippopotamus stands in the way of the acceptance of the above-mentioned hypothesis of a cholesterol cycle, or indeed of any other hypothesis on cholesterol and bile in relation to digestion. It seemed therefore well worth while to subject the bile of this animal to closer investigation. For this purpose it was deemed necessary to obtain the materials from a healthy animal in a natural state.

For the specimens we are indebted to the kindly interest of the Under-Secretary of State for the Colonies and his staff and the Governor of Kenya. The specimens were taken from an adult male shot on the lower Tana river by Mr Keith Caldwell in March, 1923.

Bile of the Hippopotamus.

Mr Caldwell reported that the animal killed had a very small gall bladder and that he doubted whether it contained much more than half a pint of bile. The bile was poured into excess of alcohol in a Winchester quart bottle for transit to England. The alcoholic solution was green in colour and contained a moderately bulky precipitate. The precipitate was filtered, well washed with alcohol and dried *in vacuo*. It dried up to a tough scale dark green in colour and weighing 1·1588 g. The alcoholic filtrate and washings were made up to 2 litres, and an aliquot portion (25 cc.) on evaporation gave a residue which after drying *in vacuo* weighed 0·1522 g. The total solid matter in solution was therefore 12·176 g. and the total solid matter of the whole bile 13·3348 g. If Mr Caldwell's estimate of the volume of the original bile is approximately correct the solid matter per 100 cc. would be 4·7 g. The residue obtained on evaporating the sample was soluble in warm water giving a clear yellowish

solution, with a slight green fluorescence, which was neutral to litmus. On cooling it became slightly turbid. This was added to the rest of the alcoholic solution (1975 cc.) and the bulk of the alcohol distilled off. The concentrated solution was then diluted with water and thoroughly extracted first with ether, then light petroleum and finally with ether. The combined extracts were made up to 500 cc. An aliquot portion (50 cc.) was evaporated and the residue after drying *in vacuo* to constant weight weighed 0·0836 g. This residue was dissolved in alcohol and the free cholesterol estimated by precipitation with digitonin, using the procedure of Fraser and Gardner [1910]. 0·0225 g. of cholesterol digitonide was obtained, equivalent to 0·00547 g. of cholesterol. The ether washings from the digitonide were evaporated to dryness, taken up in chloroform and the solution tested with acetic anhydride and sulphuric acid. A brown colour developed, which on standing turned a dusky green, so that possibly traces of cholesterol esters were present. The remaining ether extract (450 cc.) was evaporated, taken up in alcohol and hydrolysed by boiling with excess of an alcoholic solution of sodium ethoxide. After evaporating the greater portion of the alcohol, the product was diluted with water and extracted with ether in the usual manner. 0·1227 g. of an unsaponifiable matter was obtained. This was dissolved in alcohol and the cholesterol estimated, using in this case the procedure of Windaus [1910]. The cholesterol digitonide separated in typical crystalline flakes and weighed 0·2003 g. Thinking that owing to the volume of alcohol used a small portion of cholesterol digitonide might have been retained in solution, the filtrate was carefully evaporated to dryness, the residue washed well with ether and freed from excess of digitonin by washing with warm water. A further quantity of cholesterol digitonide weighing 0·0061 g. was obtained. The total cholesterol in the whole 500 cc. of ether extract was therefore 0·05575 g. and the free cholesterol was 0·0547 g. so that by difference the ester cholesterol was 0·001 g. This difference was probably real and not due to error of experiment, since the filtrate from the digitonide of the free cholesterol gave the sterol colour reaction with acetic anhydride and sulphuric acid. The cholesterol digitonide was dissociated in the usual manner in the vapour of boiling xylene, and after distilling off the xylene in steam the residue was crystallised from alcohol. It crystallised in the typical cholesterol plates and after twice recrystallising melted at 146–147°. The benzoate, prepared by the pyridine method, crystallised in typical form and, on heating, melted at 146° to a turbid liquid, which cleared at 170–180°, and on cooling showed the colour changes characteristic of cholesterol benzoate. The bile of the hippopotamus contains, according to Hammarsten, ethereal sulphates. This we were able to confirm, and thinking it possible that some cholesterol might be present as an ethereal sulphate, the aqueous mother liquors after extraction with ether were evaporated to dryness, dissolved in 100 cc. glacial acetic acid and boiled under a reflux for 4 hours. The solution was then largely diluted with water and thoroughly extracted with ether. The ethereal solution was freed from acid by thoroughly

washing first with water, then with alkali and finally with water. The ether was then distilled off, and the slight residue taken up in alcohol and boiled for 2 hours with sodium ethoxide. On diluting and extracting with ether in the usual way a residue weighing 0·076 g. was obtained. This yielded 0·0257 g. of cholesterol digitonide corresponding to 0·0062 g. of cholesterol. The ether washings from this digitonide were evaporated and taken up in chloroform. The solution gave with acetic anhydride and sulphuric acid a deep brown colour, which showed no further change on standing. We had not sufficient material to attempt to decide the interesting question whether this small quantity of cholesterol was really present in the bile as an ethereal sulphate or in chemical combination with some bile acid [Wieland and Weil, 1912]. The dark green solid from the alcoholic bile was rather difficult to powder. It was insoluble in alcohol but yielded to ether a trace of pigment which coloured the solution pink. Thinking that possibly some sterol matter, probably in ester form, might have been occluded in this material, it was largely dissolved by boiling under a reflux with an alcoholic solution of sodium ethoxide for several hours. The solution was then diluted with water and thoroughly extracted with ether. The washed ethereal extract on evaporation left a slight residue, which was taken up in alcohol and precipitated by digitonin, using the method of Fraser and Gardner. 0·0044 g. of cholesterol digitonide was obtained, corresponding to 0·001 g. of cholesterol.

We can generally confirm Hammarsten's description of the properties of the hippopotamus bile, with the exception of his statement about cholesterol, but the quantity at our disposal was insufficient for any attempt to extend his interesting account of the bile acids present.

Blood.

The fresh blood taken from the animal was carefully poured into excess of alcohol for transit. The proteins were precipitated in a fairly finely divided condition without any large clots, and beyond this precipitation the blood appeared to have undergone no autolytic change during the journey. It was filtered and the precipitate was well washed first with alcohol and then with ether and allowed to dry in the air on a porous tile. The air-dried material was bright red in colour and weighed 84·08 g. A small sample was dried to constant weight at 100°, and the result showed that the 84·08 g. of air-dried material contained 15·02 g. moisture.

The filtrate and washings were made up to 2200 cc. An aliquot portion (100 cc.) was evaporated on the water-bath and dried to constant weight *in vacuo*. The dry residue weighed 0·245 g. so that the total solid matter in the whole solution was 5·39 g. Assuming that the fresh blood contained 80 % of water, the weight of blood corresponding to the total dry matter would be about 372 g. 8·1887 g. of the air-dried red solid were dissolved in 2 % caustic soda solution, and the fluid repeatedly extracted with ether according to the method recommended by Fex [1921]. In this way 0·0318 g. of ether extract

was obtained. This was dissolved in alcohol and precipitated with excess of digitonin to obtain the amount of free cholesterol. 0·0074 g. of cholesterol digitonide was obtained, corresponding to 0·001799 g. of cholesterol.

To estimate the total cholesterol 9·1426 g. were similarly treated and the ether extract hydrolysed by means of sodium ethoxide in the usual way. The alcoholic solution of the unsaponifiable matter on precipitation with digitonin gave 0·0095 g. of compound corresponding to 0·002309 g. cholesterol.

84·08 g. of the air-dried red matter contained therefore 0·02124 g. total cholesterol, 0·01847 g. of free cholesterol and by difference 0·00277 g. of cholesterol in ester form.

The alcoholic filtrate, remaining after estimating total solids (2100 cc.) was evaporated to get rid of most of the alcohol, diluted with water and thoroughly extracted with ether. The ethereal solution was concentrated to 100 cc. 50 cc. on evaporation yielded 0·2889 g. of ether extract, so that the "fat" in 5·39 g. of solids was 0·6053 g. This was dissolved in alcohol and the free cholesterol precipitated in the usual manner with excess of digitonin. 0·2359 g. of compound corresponding to 0·05737 g. of cholesterol was obtained.

To estimate the total cholesterol the remaining 50 cc. were evaporated and hydrolysed with sodium ethoxide. On extraction with ether 0·1185 g. unsaponifiable matter yielded with digitonin 0·2345 g. of compound, equivalent to 0·057 g. cholesterol. Evidently this portion of the blood contained no cholesterol in ester form.

The total cholesterol in the 5·39 g. of solid matter dissolved in the alcohol was 0·119 g., all in the free state. In the whole blood the cholesterol in ester form was only 2·0 % of the total. This of course is on the assumption that no change had taken place in the ratio of free to ester cholesterol on keeping in alcohol for 6 months.

Flesh.

Pieces of muscle were cut off from the animal and placed in a stoppered jar with a little alcohol for transit. The material was cut into thin slices and minced. The meat was free from obvious fat and seemed quite sweet. As far as one could judge by the eye it did not appear to have undergone any autolytic change. The alcohol (about 40 cc.) was evaporated and the small residue added to the mince.

50·35 g. were taken for analysis. This was covered with 2 % solution of caustic soda, allowed to stand overnight, and then heated on the water-bath for half an hour, when practically the whole of the solid matter went into solution (method of Fex [1921]). This was thoroughly shaken with ether and allowed to stand 12 hours for the ether to separate. This process was repeated three times when it was found that nothing more was extracted. The combined ethereal solutions were evaporated and the residue dried to a constant weight. 0·1032 g. of ether extract, *i.e.* 0·205 % was obtained.

This ether extract was dissolved in alcohol and the free cholesterol pre-

cipitated with excess of digitonin. 0·114 g. compound, corresponding to 0·0277 cholesterol, was obtained. The ether washings of this compound were evaporated and the residue, hydrolysed by means of sodium ethoxide in the usual manner, yielded 0·0201 g. of unsaponifiable matter. This on precipitation with digitonin gave 0·0084 g. of compound, equivalent to 0·00204 g. cholesterol in ester form.

The alkaline liquors left after extraction of the original stuff with ether were acidified and again extracted with ether. A minute trace of crystalline matter was obtained, but it contained no cholesterol.

The percentage of cholesterol in ester form was 6·8 of the total.

The results are summarised in the following table:

	Bile			Blood		Flesh
	Wt. in g. for whole bile	As % of the total solids	Per 100 cc. of bile*	% of total dry matter	Per 100 g. of blood assuming 80 % water	% of material (as received)
Ether extract	0·836	6·27	0·298	—	—	0·205
Total cholesterol	0·063	0·472	0·0224	0·1892	0·0379	0·059
Free cholesterol	0·0547	0·410	0·0195	0·1854	0·0372	0·055
Ester cholesterol	0·0083	0·062	0·0029	0·0037	0·0007	0·004

* Assuming Mr Caldwell's estimate is correct.

Conclusions.

(1) The bile of the hippopotamus, as Hammarsten found, is poor in fat. It contains, however, cholesterol both in the free and ester forms. In the specimen examined the total cholesterol was 0·47 % of the total solid matter.

The portion of the bile solids soluble in ether contained 6·54 % of free and 0·126 % of cholesterol in ester form. The portion insoluble in alcohol contained 0·001 g. of cholesterol, most probably in ester form. The part soluble in water and alcohol contained 0·0062 g. of cholesterol most probably in the form of an ethereal sulphate, since it could not be extracted by ether, and the cholesterol was only liberated by acid hydrolysis. This constituted about 74 % of the total ester cholesterol. The percentage of total cholesterol in this bile is much lower than the values given for the bladder bile of domestic animals such as the cow; data are, however, wanting for comparison with animals at grass.

(2) The quantities of free and ester cholesterol found in the blood and in the flesh, though on the low side, are we believe within the limits found for grass-fed animals, though available data are as yet scanty.

I desire to express my sincere thanks to the Under-Secretary of State for the Colonies and his Staff at the Colonial Office, and to the Governor of Kenya for their kindness in arranging to procure the specimens: also particularly to Mr Keith Caldwell, Big Game Warden at Nairobi for his trouble in finding and shooting a suitable animal and for the great care he took in cutting out, preserving and packing the specimens for transit. I also have to thank the Government Grant Committee of the Royal Society for help in defraying the laboratory expense of the investigation.

REFERENCES.

Dorée and Gardner (1908, 1). *Proc. Roy. Soc. London*, B, **80**, 212.
—— —— (1908, 2). *Proc. Roy. Soc. London*, B, **80**, 227.
—— —— (1909). *Proc. Roy. Soc. London*, B, **81**, 111.
Ellis and Gardner (1909, 1). *Proc. Roy. Soc. London*, B, **81**, 129.
—— —— (1909, 2). *Proc. Roy. Soc. London*, B, **81**, 505.
—— —— (1912, 1). *Proc. Roy. Soc. London*, B, **84**, 461.
—— —— (1912, 2). *Proc. Roy. Soc. London*, B, **85**, 385.
—— —— (1912, 3). *Proc. Roy. Soc. London*, B, **86**, 13.
Fox (1921). *Biochem. Z.* **104**, 82.
Fraser and Gardner (1909). *Proc. Roy. Soc. London*, B, **81**, 230.
—— —— (1910). *Proc. Roy. Soc. London*, B, **82**, 559.
Gardner (1921). *Biochem. J.* **15**, 251.
Gardner and Fox (1921). *Proc. Roy. Soc. London*, B, **92**, 358.
Gardner and Lander (1913). *Biochem. J.* **7**, 576.
Hammarsten (1911). *Z. physiol. Chem.* **66**, 4, 123.
Kosumoto (1908). *Biochem. Z.* **14**, 411.
Mueller (1915). *J. Biol. Chem.* **22**, 1.
Thannhauser (1923). *Deutsch. Arch. klin. Med.* **141**, 290.
Wacker and Hueck (1913). *Arch. exp. Path. Pharm.* **74**, 416.
Wieland and Weil (1912). *Z. physiol. Chem.* **80**, 287.
Windaus (1910). *Z. physiol. Chem.* **65**, 110.
Windaus and Neukirschen (1920). *Ber. deutsch. chem. Ges.* **52**, 1915.

C. MAMMARY SECRETION. V.

I. FURTHER RESEARCH ON THE THRESHOLD AND EFFECTS OF PROTEIN "EXCESS."

II. THE QUANTITATIVE RELATION OF VITAMIN B TO PROTEIN.

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In a previous paper [Hartwell, 1922, 1] it was shown that the proportion of 1 g. protein (egg albumin or caseinogen) to 15 g. bread, in a mother rat's diet, was sufficient to cause abnormal symptoms in the sucklings. In these experiments small quantities of yeast extract (marmite) were added to improve the palatability of the diet. At that time the significance of vitamin B was not understood, and therefore no effort was made to use the same supply of yeast extract throughout the work. In the light of more recent experiments, therefore, it was thought advisable to repeat some of this earlier work, omitting yeast extract entirely. It was also hoped by a longer series of experiments, using other proportions of protein as well, to ascertain where the greatest danger level lies.

The second group of experiments described in this paper was started with a view to finding if a quantitative relation existed between the protein and vitamin B in the lactating rat's diet. In some previous experiments [Hartwell, 1922, 2] it was shown that 50 cc. of various fruit (except grapes) and vegetable juices, when added to the mother's "excess" protein diet, prevented any bad symptoms in the sucklings. The different juices did not give equally good results, and as fruits and vegetables are recognised as containing varying quantities of vitamin B, it seemed possible that some quantitative relation might exist between the protein and the vitamin B. In these experiments [Hartwell, 1922, 2] the amount of caseinogen was kept constant and it was noticeable that the best results were obtained by the addition of juices (or other foods, *e.g.* egg yolk) shown by other workers to be especially rich in vitamin B.

With our present state of knowledge, any quantitative work on vitamins is very difficult, especially so, as no methods for the complete isolation of these

food factors are available. Various assumptions as to amounts needed, etc. are made, but there is little actual proof, and practically no accurate work on the subject.

Osborne and Mendel [1922] suggest that there is a correlation between the size (and therefore the age) of the animal (rat) and its vitamin B requirement. They describe experiments indicating that on a definite and low vitamin B intake the heavier animals fare worse than those of lighter weight. These workers used one diet throughout, and themselves point out that their experiments throw no light on the question as to whether the composition of the food has any direct relation to the vitamin B intake.

Cowgill [1923], working on dogs, found a similar relation between the vitamin B intake and the animal's body weight.

Following along these lines of argument it might be expected that the requirements of the lactating animal, in respect of vitamin B, would exceed those of the non-lactating creature. Other workers have assigned to vitamin B an important rôle in connection with glandular secretion, and, if this is correct, an extra supply of this vitamin would be needed by the active mammary gland.

Experimental data.

(*a*) *Method.* During gestation the mother rats received a good mixed diet consisting of kitchen scraps with a small amount of bread and milk per day. At least three animals were fed on each diet, and each attempted to rear six babies (with the exception of 1177 and 1199, where the litters consisted of five only). In previous experiments the litters have been reduced to six on the first day. A modification of this procedure has recently been adopted, viz. the litter was not reduced in number until the second day. It was noticed that large litters (12 or more) frequently contained one or two weakly members which either died or were eaten by the mother within 24 hours; hence a better selection could be made on the second day and there was less risk of losing a baby during the first few days of lactation. The mothers and litters were weighed every day as previously described [Hartwell, 1921, 1], and the foods were given in the form of a paste, all constituents being well mixed and increased proportionately when necessary. The tomato juice and the extract from marmite rich in vitamin B were prepared as described in an earlier paper [Hartwell, 1922, 2].

(*b*) *Experiments.* A brief summary of the experiments is given below:

1. *Experiments with bread, "food casein"* (82·86 % *protein*) [*Hartwell*, 1922, 1] *and water* (Fig. 1).

Diet. 15 g. bread to x g. food casein mixed to a paste with water.

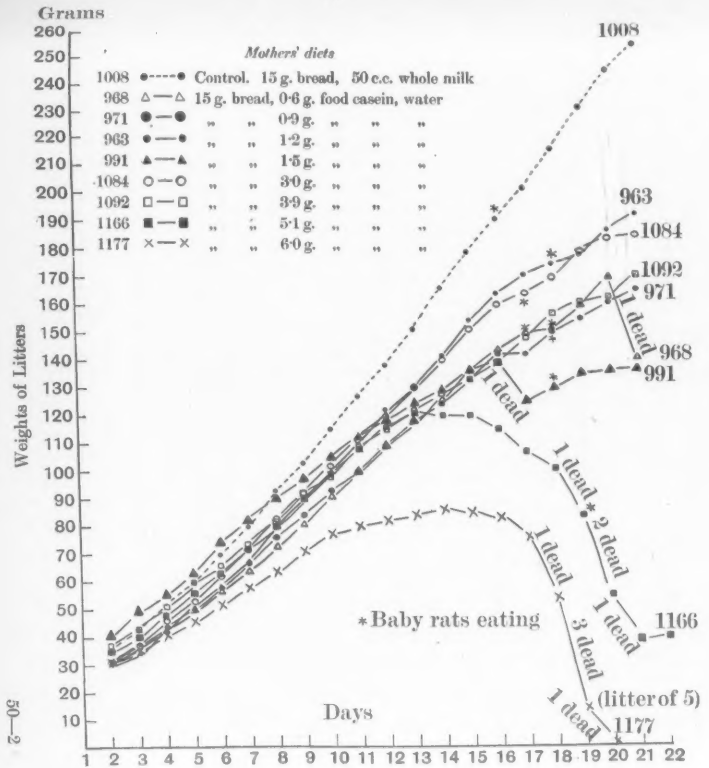
Grams
Mothers' diets
1008 Control. 15 g. bread, 50 c.c. whole milk
968 15 g. bread, 0·6 g. food casein, water
971 „ „ 0·9 g. „ „ „
963 „ „ 1·2 g. „ „ „
991 „ „ 1·5 g. „ „ „
1084 „ „ 3·0 g. „ „ „
1092 „ „ 3·9 g. „ „ „
1166 „ „ 5·1 g. „ „ „
1177 „ „ 6·0 g. „ „ „
Weights of Litters
Days
*Baby rats eating
1 dead
2 dead
3 dead
(litter of 5)
1008
963
1084
1092
971
968
991
1166
1177

Fig. 1.

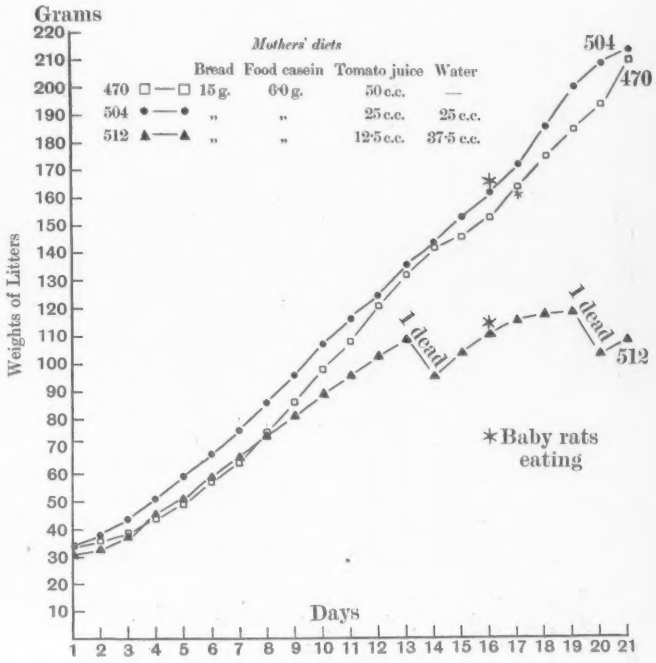
Grams
Mothers' diets
Bread Food casein Tomato juice Water
470 15 g. 6·0 g. 50 c.c. —
504 „ „ 25 c.c. 25 c.c.
512 „ „ 12·5 c.c. 37·5 c.c.
Weights of Litters
Days
*Baby rats eating
1 dead
504
470
512

Fig. 2.

Proportion of food casein to 15 g. bread	Litter numbers	Results
Expt. A.		
0·6 g.	962 968 975	No spasms or screaming fits—babies not quite normal—17th–18th days they appeared frightened and slightly jerky in their movements—3 out of 18 died.
Expt. B.		
0·9 g.	969 970 971	All 3 litters showed spasms—one lot had screaming fits and one litter could not be weaned until 27th day, instead of the 21st as usual—3 out of 18 died—rate of growth of young about the same as when the mother received 0·6 g.
Expt. C.		
1·2 g.	963 964 965	All 3 litters exhibited spasms and screaming fits—3 out of 18 died—rate of growth of young slightly better than when the mother had less protein, but general condition of litters worse.
Expt. D.		
1·5 g.	991 994 995	Condition practically the same as with 1·2 g. but rate of growth less good at end of lactation—3 out of 18 died.
Expt. E.		
3·0 g.	1070 1084 1085 1087 1088 1089 1157 1159 1164	Condition rather better than with lower proportions of protein—4 out of 54 died.
Expt. F.		
3·9 g.	1091 1092 1093	All 3 litters showed typical symptoms and one whole litter died, *i.e.* 6 out of 18—growth about the same as before.
Expt. G.		
5·1 g.	1165 1166 1173	Condition much worse—11 out of 18 died (this involves one entire litter).
Expt. H.		
6·0 g.	1111 1177 1199	None survived.

In all these experiments the rate of growth of the litters for the first 12 days was quite good (except when the mother had the proportion 6 g. food casein) and fairly constant in spite of the variation in the amount of protein fed to the mother. The six young on the 12th day weighed approximately 110–120 g. With the lower proportions of protein the babies weighed about 30 g. each at weaning on the 21st day, while with the higher proportions they were less, showing only slight gains after the 12th day. The controls usually weigh 40–45 g. at weaning. The mothers remained well throughout in spite of the fact that many of them lost weight. It was noticeable that an appreciable loss in weight of the mother was usually correlated with a better condition in the sucklings. This point has been commented upon in a previous communication [Hartwell, 1921, 2].

From these experiments it is obvious that even small quantities of protein

(0·6 to 15 g. bread) added to a bread diet result in the suckling rats exhibiting an abnormal condition. With an increase in the protein ration the symptoms became gradually worse, and when the mother rat was given 6 g. food casein to 15 g. bread, none of the babies survived. The supply of vitamin B was practically constant and therefore it seemed probable that if the vitamin B were increased with the protein, the bad effects might be obviated. The following experiments were started with a view to testing this hypothesis.

2. *Quantitative relation of protein to vitamin B.*

(*a*) Experiments using tomato juice as a source of vitamin B.

Expts. I and J. These are comparable with Expt. C (p. 788) for the animals were receiving the same proportion of bread and food casein, but tomato juice was added.

Diet. 15 g. bread, 1·2 g. food casein, 1·5 cc. tomato juice, 31·5 cc. water.

972 One litter showed spasms. All 3 litters grew well and their coats were good, but from
973 about the 18th day (*i.e.* when the baby rats ran about and ate for themselves) they
974 appeared frightened and exhibited slight incoordination.

Thus the condition of the sucklings was improved by adding 1·5 cc. of tomato juice to the mother's initial diet, but the bad effects were by no means obviated.

Diet. 15 g. bread, 1·2 g. food casein, 3 cc. tomato juice, 30 cc. water.

979 Growth of the young was rather better than in Exp. I but spasms and incoordination
980 were still exhibited, although not quite so markedly.
981

Expt. K. Comparable with Expt. D (p. 788).

Diet. 15 g. bread, 1·5 g. food casein, 6 cc. tomato juice, 27 cc. water.

1151 The baby rats were normal in all respects, and the growth curve was quite good. Their
1153 fur was especially thick.
1156

Hence it appears that 6 cc. of tomato juice when added to the mother's diet of 15 g. bread and 1·5 g. food casein contained sufficient vitamin B to prevent any abnormal symptoms in the suckling rats.

Expts. L, M, N, O. Comparable with Expt. H (p. 788).

Diet. 15 g. bread, 6 g. food casein, 6 cc. tomato juice, 45 cc. water.

1345 The growth of the young was less good than when a lower proportion of protein was fed
1352 to the mother rat. Spasms etc. were evident from the 10th day and onwards and the
1353 baby rats were not normal for some days after weaning.

Diet. 15 g. bread, 6 g. food casein, 12·5 cc. tomato juice, 37·5 cc. water (Fig. 2).

512 The results were similar to those described for Expt. L. It might be expected that
516 Expt. M should afford better results than Expt. L, but in the latter the mothers lost weight
517 and in the former they did not. It has been pointed out previously [Hartwell, 1921, 2] that a loss in weight of the mother frequently means a better condition in the offspring.

Diet. 15 g. bread, 6 g. food casein, 25 cc. tomato juice, 25 cc. water (Fig. 2).

492
493
504

Diet. 15 g. bread, 6 g. food casein, 50 cc. tomato juice, 25 cc. water (Fig. 2).

470
472
473

In both cases the young were normal in all respects and the growth was good, being practically the same as that obtained in Expt. K.

Expt. P.

Diet. 15 g. bread, 21 g. food casein, 111 cc. tomato juice.

1013 No bad symptoms were seen in any litter; the growth was not quite so good as in
1014 Expts. K, N, O.
1015
1130
1138
1142

(*b*) Experiments using extract from "marmite" as a source of vitamin B.

Expts. Q and R to compare with Expts. C and K.

Diet. 15 g. bread, 1·5 g. food casein, 0·6 cc. extract containing vitamin B, 33 cc. water (Fig. 3).

1244 Growth of the young rats was good and their condition normal.
1262
1280

Diet. As above, but 0·9 cc. extract instead of 0·6 cc. (Fig. 3).

1238 The results were the same as those in Expt. Q, therefore the increase in the vitamin B
1242 content of the mothers' diet had no apparent effect.
1279

Expts. S and T to compare with Expt. H (p. 788).

Diet. 15 g. bread, 6 g. food casein, 0·9 cc. extract, 48 cc. water (Fig. 3).

1307 All 3 litters suffered badly, and spasms etc. were noted from the 13th day and onwards.
1308 The growth of the sucklings was fairly good up to the 12th day, but after that it was
1309 *very* slow.

Therefore it may be concluded that 0·9 cc. of the extract was not sufficient when the protein ration was increased.

Diet. 15 g. bread, 6 g. food casein, 3 cc. extract, 45 cc. water.

Condition normal [see Hartwell, 1922, 2, litter 396].

Expt. U to compare with Expt. P.

Diet. 15 g. bread, 21 g. food casein, 12 cc. extract, 99 cc. water.

1147 The sucklings grew very slowly, but no abnormal symptoms were observed.
1150
1243

(*c*) Experiments with bread and tomato juice.

Expt. V.

Diet. 15 g. bread, 33 cc. tomato juice (Fig. 4).

1019 A slow, but steady growth curve was given by the sucklings, but the mothers' curves
1020 show an appreciable loss of weight, probably due to the low protein content of the diet.
1021

(*d*) Experiments with bread, starch and salt mixture (Fig. 4).

Diet. 15 g. bread, 5 g. starch, 0·7 g. salt mixture [Hartwell, 1922, 1].

1002 The results were practically the same as described for Expt. V, *i.e.* bread + excess
1003 vitamin B; therefore starch does not need extra vitamin B, whereas protein does.
1011

Consideration of results.

In the first series of experiments the diet consisted of bread, food casein and water. Such a diet is low in fat and probably also in vitamin A, since these constituents were only present (if at all) in small quantities in the food casein. Vitamin B might also be present in the protein used, but the chief source of

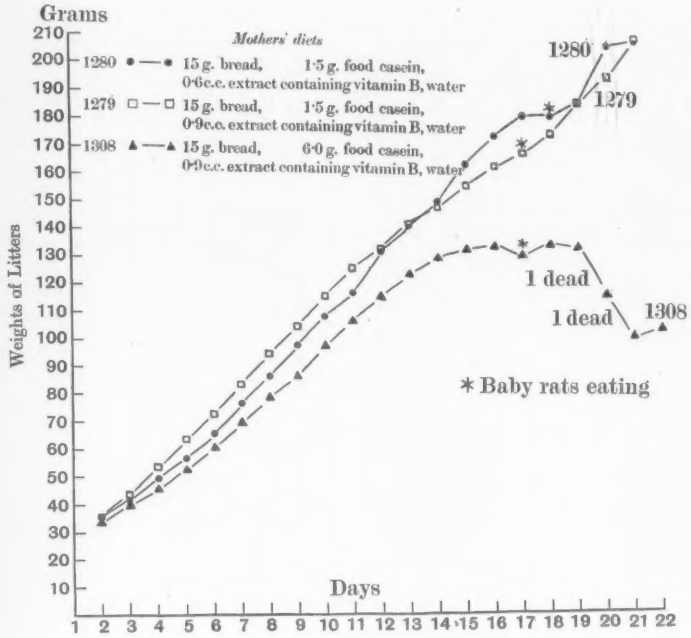
Grams
Weights of Litters
Mothers' diets
1280 ●—● 15 g. bread, 1·5 g. food casein, 0·6 c.c. extract containing vitamin B, water
1279 □—□ 15 g. bread, 1·5 g. food casein, 0·9 c.c. extract containing vitamin B, water
1308 ▲—▲ 15 g. bread, 6·0 g. food casein, 0·9 c.c. extract containing vitamin B, water
1280
1279
1 dead
1 dead
1308
* Baby rats eating
Days

Fig. 3.

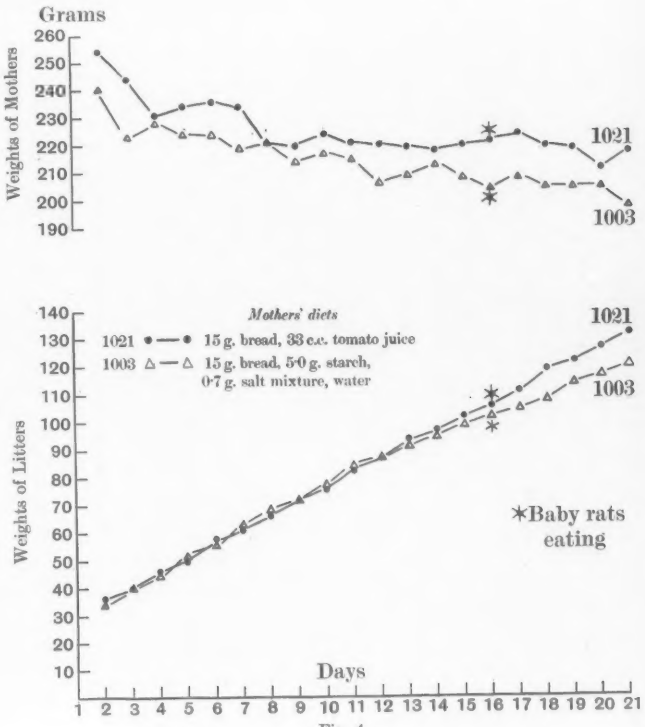
Grams
Weights of Mothers
1021
1003
Mothers' diets
1021 ●—● 15 g. bread, 33 c.c. tomato juice
1003 △—△ 15 g. bread, 5·0 g. starch, 0·7 g. salt mixture, water
1021
1003
*Baby rats eating
Weights of Litters
Days

Fig. 4.

it is the baker's bread, which has been shown to contain enough vitamin B to provide for the growth of the rat for several months ($7\frac{1}{2}$ months in one experiment) [Hartwell, 1924]. When 0·6 g. of food casein was added to 15 g. of bread in the mother's diet, the babies were not quite normal, while the addition of 0·9 g. food casein to 15 g. of bread led to the sucklings exhibiting spasms and screaming fits. With further increases in the protein in the mother's diet, the babies became gradually worse, until finally, when 6 g. of food casein were added, none survived.

Drummond, Crowdon and Hill [1922] state that they have observed symptoms in suckling rats similar to those described by me, but "only in the case of those being reared by mothers on diets deficient in vitamin B."

In some unpublished work of the author's, rats were kept for 9 months on a diet of 30 g. bread, 3 g. food casein, 3 g. butter, 0·7 g. salt mixture, and water; in another experiment rats were kept for 4 months on a similar diet but with a higher proportion of protein, viz. 12 g. food casein instead of 3 g. In both experiments the animals remained fit and healthy and never exhibited any symptoms indicative of lack of vitamin B. Therefore such diets cannot be said to be deficient in vitamin B. It might be suggested that there is not much excess of vitamin B in bread and that the addition of other constituents to a bread diet would necessarily dilute the vitamin B in the diet, and thus put the vitamin B content below the limit required for health. That this is not so is shown by the experiments described in this paper in which the mother rats received a ration of 15 g. bread, 5 g. starch, 0·7 g. salt mixture, and water, and the babies were successfully reared and showed no spasms. The mother rats also remained in good condition. Hence a diet of 15 g. bread + 5·7 g. extra solid is *not* deficient in vitamin B; further, the spasms were noticed when the mother rat received only 0·9 g. of food casein to 15 g. bread.

Two sets of mother rats were fed respectively on 15 g. bread, 5 g. starch, 0·7 g. salt mixture, and 15 g. bread and 33 cc. tomato juice. Examples of the weight curves of the mothers and litters are shown in Fig. 4, and it is interesting to note how nearly they coincide. In both, the growth of the baby rats is slow, owing to the low proportion of protein in the mothers' diets. Since the mothers receiving tomato juice must have had an excess of vitamin B, and the growth curves of the sucklings are practically equal, it does not appear that there is any lack of vitamin B in the diet of bread, starch and salt mixture.

Again Drummond, Crowdon and Hill state that "A high protein diet can be adequate for lactating female rats...provided that it is also adequate in components serving as sources of the vitamins." It is difficult to know what is meant by "adequate" and also although these authors say vitamins, they suggest no possible function for either vitamin A or vitamin C in this connection.

The experiments described in this paper afford evidence that there is a distinct *quantitative* relation between protein and vitamin B in the diet of a lactating rat, *i.e.* the more protein in the diet, the more vitamin B must be added for the offspring to be successfully reared. An excess of vitamin B has

no effect, for the addition of 50 cc. of tomato juice instead of 25 cc. to a diet of 15 g. bread and 6 g. food casein made no apparent difference either to the growth curve or general condition of the young. Similar results were obtained when 0·6 cc. or 0·9 cc. of extract of vitamin B were added to a diet of 15 g. bread and 1·5 g. food casein.

It is interesting that the growth curves of the young are practically the same when the mother receives 1·5 g. or 6 g. food casein to 15 g. bread provided that more vitamin B is added in the latter instance.

The exact amounts of vitamin B necessary for different levels of protein intake have not yet been determined, but experiments are in progress to elucidate this point. It is quite possible, almost probable, in fact, that the amount of vitamin B will not only vary with the protein intake, but will be affected by other dietary constituents.

In the experiments described in this paper the diets were very low in fat and vitamin A. If larger amounts of fat were present it is conceivable that even more vitamin B might be needed to prevent spasms. This low proportion of fat did not seem to affect either the mothers or the offspring adversely, possibly because the mother rats were in very good condition and had fairly large amounts of depôt fat.

Drummond, Crowdon and Hill [1922] state also that there is some evidence for believing that vitamin B is concerned with carbohydrate metabolism, since the non-nitrogenous residue from amino acids is said to be metabolised along the same path as carbohydrates. They suggest therefore that this explanation might be applied to my results. That this is not so, is quite obvious from my earlier work [Hartwell, 1921, 1]. No spasms were developed when the mother rats received 15 g. bread and 5 g. dextrin, and if extra vitamin B were needed for the proper metabolism of the carbohydrate, surely spasms should have resulted from this large addition of carbohydrate to the diet. These results have been confirmed by feeding the mother rats on 15 g. bread, 5 g. starch and 0·7 g. salt mixture, when no spasms developed in the baby rats. In this earlier paper [Hartwell, 1921, 1] it was also shown that adding 5 g. butter to 15 g. bread produced no harmful results in the suckling babies, which proves that vitamin B is not specially concerned, in the lactating animal, with fat metabolism; hence it appears fairly clear that the rôle of vitamin B in the metabolism of protein is concerned with the nitrogenous part of the protein molecule or with protein quâ protein, and not with any fatty acid or carbohydrate residue.

Conclusions.

1. In the lactating rat, when protein is added to a diet of bread, as little as 0·6 g. food casein (82·86 % protein) to 15 g. bread is sufficient to produce an abnormal condition in the young. Increasing the proportion of protein in the mother's diet leads to worse symptoms in the sucklings, and when 6 g. of this protein are added to 15 g. bread none of the young survives.

2. It is impossible to state what constitutes an excess of protein in a lactating rat's diet because there is a *quantitative* relation between protein and vitamin B. Thus, provided the vitamin B is increased when the protein is increased there should be no evil effects.

3. In the lactating rat the function of vitamin B is primarily concerned with the metabolism of protein quâ protein or with the nitrogen part of the protein molecule; for to a diet of 15 g. bread and 5 g. protein must be added far more vitamin B than to a diet of 15 g. bread and 5 g. starch.

4. A high proportion of protein (15 g. bread to 21 g. food casein) in the lactating rat's diet produces no harmful effects in the offspring, provided the diet is very rich in vitamin B, but the rate of growth of the sucklings is impaired.

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REFERENCES.

Cowgill (1923). *J. Biol. Chem.* **56**, 725.
Drummond, Crowdon and Hill (1922). *J. Physiol.* **56**, 413.
Hartwell (1921, 1). *Biochem. J.* **15**, 140.
—— (1921, 2). *Biochem. J.* **15**, 563.
—— (1922, 1). *Biochem. J.* **16**, 78.
—— (1922, 2). *Biochem. J.* **16**, 825.
—— (1924). *Biochem. J.* **18**, 120.
Osborne and Mendel (1922). *J. Biol. Chem.* **54**, 739.

CI. ON THE SPECIFIC ACTION OF PLANT PROTEASES

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The problem of the specific action of ferments which is one of great general importance for biology in its application to plant proteases has a comparatively short history. Aron and Klempin [1908] and Bialosuknia [1909] were the first to detect that the ferment preparations from the seeds of wheat, rye and oats acted with great activity on proteins of cereals and vetch. The same preparations were without action on proteins of animal origin. Jacobson [1912] stated that the proteases of alfalfa seeds hydrolysed caseinogen and Witte-pepton, but left egg-albumin, serum-albumin, legumin and conglutin intact. At about the same time B. Issatschenko [1917] published the results of his investigations on the specific proteoclastic action of antibodies, which are produced in the organism of the rabbit by parenteral injection of various plant proteins. The application of Abderhalden's ninhydrin reaction showed that the proteoclastic action was strictly specific and antibodies of the proteins from flax seed, wheat, hazel-nut and oats were active only in relation to their own proteins, but not to the others. My present experiments give a new confirmation of these facts.

In order to isolate proteins from seeds I used extraction with 5 % NaCl solution at 60° (10 litres of solution for 1 kilo. of ground seeds). After filtration the globulins were precipitated by diluting the sodium chloride extract with pure water. After having been filtered and washed with water, the globulins were treated with alcohol and ether or with acetone and placed in a vacuum exsiccator over sulphuric acid.

As ferment preparations were taken, except in the first series of experiments, the ground seedlings of the same plants as gave the material for the extraction of the globulins. Sörensen's formaldehyde titration method was employed for quantitative determination of the amino-groups, liberated in the process of protein hydrolysis. The results are presented either as milligrams of amino-nitrogen or in the form of the so-called amino-index (the ratio of the total nitrogen of the protein to its amino-nitrogen). All the experiments were parallel and the results given in the tables present the average of two determinations.

In the first series of experiments the proteins were dissolved in 5 % NaCl solution. For the ferment preparation the fresh seedlings were ground in a mortar with the same NaCl solution, pressed and filtered.

For the determination of the influence of NaCl on proteases the following control experiment was carried out. Seedlings of *Phaseolus Mungo* L., aged one day, were divided into eight equal parts, ground and diluted each with 40 cc. of solvent (water or 5 % NaCl solution). Two samples of each group were titrated by Sörensen's method immediately, two others kept with toluene at room temperature and titrated after 24 hours. The divergence of results in each series was within the limits of possible experimental error.

	Amino-nitrogen in mg.					
	Initial			After 24 hours at room temperature		
	1	2	Average	1	2	Average
Water	15·0	14·6	14·8	24·3	25·2	24·8
5 % NaCl solution	15·0	15·0	15·0	23·7	24·5	24·7

Exp. 1. The press-juice of seedlings of *Cannabis sativa* L. as ferment. The globulins of *Cannabis sativa* and other plants, 1 g. for each sample. Toluene as antiseptic. The acidity in all flasks only showed a very small difference at the end of the experiment: no greater than 2 cc. of $N/5$ NaOH for 50 cc. of reacting liquid. The globulins without ferment after standing showed no distinct change.

	Amino-nitrogen in mg.	
	Initial	Final
Globulin of *Cannabis sativa*	1·8	1·8
„ *Brassica rapa*	3·6	3·6
„ *Lupinus luteus*	3·8	3·8
„ *Phaseolus Mungo*	2·1	2·1

The content of amino-nitrogen in the ferment changed at the same time from 14·3–25·2 mg.

	Amino-nitrogen in mg.			
	Initial	Final	Calculated*	Difference
Cannabis ferment + *Cannabis* globulin	16·1	30·1	27·0	3·1
„ „ + *Brassica* „	17·9	28·8	28·8	—
„ „ + *Lupinus* „	18·1	29·0	29·0	—
„ „ + *Phaseolus* „	16·4	27·3	27·3	—

* From data for globulin and ferment separately.

The preference for its own protein by the ferment is evident.

Exp. 2. The ferment from seedlings of *Phaseolus Mungo* L.

The amounts of globulins were greater than in the first experiment. The duration of the experiment was 65 hours at 26–28°. The globulins without ferment did not change. The amino-nitrogen content of the ferment solution changed from 9·5–16·5 mg.

	Amino-nitrogen in mg.			
	Initial	Final	Calculated	Change
Phaseolus ferment + *Phaseolus* globulin	15·7	33·4	22·7	10·7
„ „ + *Cannabis* „	13·4	28·0	20·4	7·6
„ „ + *Lupinus* „	13·6	24·7	20·6	4·1
„ „ + *Brassica* „	14·1	23·0	21·1	1·9

Exp. 3. The ferment from seedlings of *Brassica rapa* L.

The conditions of the experiments were the same as in Exp. 2. The amino-nitrogen content of the ferment solution changed from 3·1–4·4 mg.

	Amino-nitrogen in mg.			
	Initial	Final	Calculated	Change
Brassica ferment + *Brassica* globulin	7·7	13·4	9·0	4·4
„ „ + *Lupinus* „	7·2	11·7	8·5	3·2
„ „ + *Phaseolus* „	9·3	10·6	10·6	—

Exp. 4. The ferment from seedlings of *Lupinus luteus* L.

The conditions were the same as in Exps. 2 and 3. The amino-nitrogen content of the ferment solution changed from 18·4–22·0 mg.

	Amino-nitrogen in mg.			
	Initial	Final	Calculated	Change
Lupinus ferment + *Lupinus* globulin	22·5	34·0	26·1	7·9
„ „ + *Phaseolus* „	24·2	33·9	28·2	5·7
„ „ + *Cannabis* „	22·3	28·9	25·9	3·0

Assuming that the maximal action for each ferment is equal to 100, the values of the other amounts are as follows:

	Globulins			
Ferments	*Phaseolus*	*Lupinus*	*Brassica*	*Cannabis*
Phaseolus	100	38	18	71
Lupinus	72	100	—	38
Brassica	0	73	100	—
Cannabis	0	0	0	100

These values indicate clearly that the proteases of various plants, acting on the different globulins, hydrolyse them to an unequal extent.

The second series of experiments gives a new confirmation of this specificity. All the globulins were newly prepared. The total nitrogen content was determined (by Kjeldahl's method) and the ratio of total nitrogen to amino-nitrogen (amino-index). The results of these determinations are given for air-dried globulins.

Plant	Total nitrogen %	Amino-nitrogen %	Amino-index
Hibiscus esculentus	13·8	0·518	26·3
Cannabis sativa L.	15·54	0·196	79·3
Dolichos melanophthalmus DC.	13·75	0·552	25·0
Lathyrus sinensis	14·94	0·515	29·1
Phaseolus Mungo L.	12·76	0·445	28·8

The samples of ferments and globulins in all experiments were strictly of 1 g. To each flask were added 30 cc. of 4 % NaCl solution and chloroform and toluene as antiseptics. The duration of all experiments was 168 hours at room temperature (19°).

Exp. 5. The ferment of seedlings of *Phaseolus Mungo* L.

The amino-nitrogen content in the ferment solution changed from 2·16–4·9 mg.

Origin of globulins	Amino-nitrogen in mg.	Amino-index		Extent of ferment action
		Initial	Final	
Phaseolus Mungo	11·5	28·8	19·03	100
Dolichos melanophthalmus	13·2	25·0	16·6	99
Hibiscus esculentus	11·3	26·3	21·2	57
Cannabis sativa	7·0	79·3	74·0	20
Lathyrus sinensis	10·1	29·1	28·7	4

Exp. 6. The ferment of seedlings of *Hibiscus esculentus*.
The amino-nitrogen content of the ferment at end of autolysis was 8·85 mg.

Origin of globulins	Amino-nitrogen in mg.	Amino-index		Extent of ferment action
		Initial	Final	
Hibiscus esculentus	20·3	26·3	11·9	100
Dolichos melanophthalmus	19·1	25·0	13·5	84
Lathyrus sinensis	14·9	29·1	24·9	26

Exp. 7. The ferment of seedlings of *Dolichos melanophthalmus* DC.
The amino-nitrogen content of the ferment at end of autolysis was 5·3 mg.

Origin of globulins	Amino-nitrogen in mg.	Amino-index		Extent of ferment action
		Initial	Final	
Dolichos melanophthalmus	14·8	25·0	14·4	100
Lathyrus sinensis	11·9	29·1	22·6	53
Phaseolus Mungo	11·5	28·8	20·6	67

Exp. 8. The ferment of *Cannabis sativa* L.
The amino-nitrogen content of the ferment at end of autolysis was 2·1 mg.

Origin of globulins	Amino-nitrogen in mg.	Amino-index		Energy of ferment action
		Initial	Final	
Cannabis sativa	7·55	79·3	28·7	100
Lathyrus sinensis	7·05	29·1	30·4	0
Hibiscus esculentus	8·4	26·3	21·6	44

The results of all experiments are summarised in the following table, similar to that for the first series:

Ferments	Globulins				
	Phaseolus	*Dolichos*	*Cannabis*	*Hibiscus*	*Lathyrus*
Phaseolus	100	99	20	57	4
Dolichos	67	100	—	—	53
Cannabis	—	—	100	44	0
Hibiscus	—	84	—	100	26

Though these experiments are preliminary to a detailed investigation, I am able to state that in all cases the ferment from every plant splits its own globulin more actively than the globulins of other plants. The close systematic relation of *Phaseolus* and *Dolichos* is clearly reflected in the mutual relation of their ferments and globulins. If further investigations confirm this regularity, observed in a large number of cases, Roaf's [1909] ideas on the connection of the specificity of enzyme action with the degree of development of a living organism may receive quite a new direction: for we may think that both proteins and enzymes are undergoing the same evolutionary process that causes the evolution of physiological properties in plants or animals as a result of deep changes in their internal cellular structure. Comparative physiology, whose development is quite recent, gives new evidence of an intimate connection between the structure and the physiological properties of an organism. Thus the question of the origin of systematic units seems to be reduced to a purely chemical problem of the evolution of proteins and other chemical bodies in the protoplasm.

REFERENCES.

Aron and Klempin (1908). *Biochem. Z.* **9**, 163.
Bialosuknia (1909). *Z. physiol. Chem.* **58**, 487.
Issatschenko (1917). *Deutsch. med. Wochensch.* No. 28.
Jacobson (1912). *J. Amer. Chem. Soc.* **37**, 1730.
Roaf (1909). *Biochem. J.* **3**, 462.

CII. ON THE NATURE OF FOETAL RE-ABSORPTION.

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Introductory.

During recent years it has been conclusively shown that following mortality *in utero* a dead foetus may be removed in one or both of two ways. First, and best known, is the process of abortion where a premature parturition entirely clears the uterus. In this case, however, the pregnancy is terminated and in polytocous animals abortion of a dead foetus could only proceed by the sacrifice of the whole litter. In such mammals, therefore, it would not be unexpected to find some other means of removing a dead embryo, and it is certain that in some animals the death of one embryo of a litter is not followed by abortion, in spite of the fact that the embryo disappears. From the fact that atrophic conceptuses in all stages of degeneration may be found in company with normal ones in most polytocous pregnancies, some kind of intra-uterine lysis and re-absorption is inferred.

A provision of this nature would avoid the uneconomic removal of living foetuses in the removal of a dead one higher up the uterus, and it may be said that the *a priori* probability is found to obtain in practice, namely, that in general, abortion occurs in monotocous animals and re-absorption in multiple pregnancies.

There is strong evidence, however, that re-absorption may occur in the early stage of single gestation [Hammond, 1921], and the process has been found to occur in such widely different mammals as mare, cow, sheep, guinea-pig, hamster, rat, mouse, ferret, dog, cat, mole, rabbit and pig [Marshall, 1922].

The number of ova ovulated at one time may be ascertained by counting the corpora lutea[1], and by using this method in conjunction with the actual enumeration of degenerate foetuses found, it has been possible to arrive at some idea of the percentage of foetuses and embryos which have died and been re-absorbed. The following table gives a compilation of the chief investigations on record:

[1] In the ordinary way one corpus luteum represents one ovum ovulated and in polytocous animals the occurrence of two ova in one Graafian follicle and of polyembryony is so rare as to be negligible. The only other source of error lies in the possibility that some ova may not be fertilised, a thing which is probably not common where the sexes are kept together [see Parkes, 1923].

Table I. *Amount of re-absorption of foetuses in various mammals.*

Author	Animal	Foetuses found: Normal	Foetuses found: Atrophic	Corpora lutea	% death and re-absorption
Hammond [1921]	Sheep	101	8	116	12·9
Hammond [1921]	Pig	267	49	396	32·5
Robinson [1921]	Ferret	1246	—	1643	24·2
Corner [1923]	Pig	3442	43	4480	23·3
Parkes [1923]	Mouse	76	—	82	7·3

With the exception of the last of these investigations the dissections were made at various stages of pregnancy and the figures do not necessarily, therefore, represent the total mortality.

A further method of considering pre-natal mortality consists in comparing the average size of litter at birth with the average corpora lutea count. Some results which have been obtained by this means are given below:

Table II. *Number of corpora lutea and size of litter at birth.*

Author	Animal	Average size of litter at birth	Average corpora lutea	% mortality
Long and Evans [1921]	Rat	6·7	10	33
Hammond [1914]	Pig	12	20	40
Robinson [1921]	Rabbit	5	9·6	37·5
Biedl, et al. [1921]	Rabbit	6·9	9·11	18·33
Robinson [1921]	Ferret	6	9·95	39·23

The second method avoids the inaccuracy involved in dissecting pregnancies in early stages where the mortality which might have occurred later cannot be ascertained, and the higher results obtained are thus explained.

The time incidence of the mortality which does not end in abortion is of considerable interest. Relative to this point Corner [1923] remarks: "...it has become apparent that defective embryos may be found in uteri which are free from infection and other pathological conditions; and that in animals which bear more than one embryo at each gestation normal and abnormal embryos may be found in the uterus at the same time. Furthermore, abnormality or degeneration of the embryo may occur at any stage of gestation." Huber [1915] for the rat and Corner [1921] for the pig, have shown that ova may become abnormal even before implantation. Corner also examined the amount of mortality which had occurred at different stages in pregnancy and found that even in pregnancies where the embryos were not larger than 40 mm. crown-rump length the missing embryos amounted to 20 %. This distribution is confirmed by Robinson's work where 16·03 % of the ova were missing by the eighth day of pregnancy.

Examination of foetuses which are undergoing degeneration and absorption reveals many points which are of importance in considering the actual nature of the process. With this end in view the few facts known regarding such degenerative conceptuses may be considered. It is known:

(1) That the degeneration is not putrefactive.

(2) That the foetus is the first part of the conceptus to disappear, and the placenta the last.

(3) That the younger the foetus, the more complete is removal.

(4) That the process appears to commence in the liver of the foetus.

(5) That the products of the degeneration are soluble.

The latest stage at which foetuses can be satisfactorily removed is a subject of some controversy, but there is no doubt that moderately young foetuses can be entirely removed. Koebner [1910] goes so far as to say that even the bony structure of later foetuses disappears completely in the rabbit under experimental conditions. Also, there appears to be no limit to the number of conceptuses which can be re-absorbed at once. It has been found experimentally that excision of the corpora lutea causes death of the embryos and complete re-absorption up to the twentieth day of pregnancy in the rabbit [Fraenkel, 1903].

Extra-uterine autolysis of foetuses.

The manner in which the foetal tissues disappear under sterile conditions points at once to the phenomena of autolysis. This indeed was the manner in which Meyer [1919] suggested this process occurred. Now it is known that nearly all the isolated adult organs if incubated at 37° under sterile conditions will in a few days become soluble and disintegrate. The chief organs in which this has been worked out are the muscles, spleen, and liver, especially the last, and the occurrence of this has been attributed to a special class of enzymes—the autolytic enzymes or tissue ferments. These enzymes are probably normal constituents of the organ but only carry out their proteolytic action on the death of the latter.

If foetal absorption is due to the action of such enzymes as these the question arises as to whether the enzymes are foetal or maternal in origin. Are they a constituent of the foetal tissues from the earliest stages, for absorption may occur even of very young embryos, or are they contained in the placenta or uterine wall? From an examination of foetuses in different stages of absorption it is found that absorption begins in the foetus (usually the liver disappears first) and terminates in the placenta which is the last to disappear. Again only dead embryos are absorbed and this also is against the autolytic enzymes being maternal in origin.

The experiments which have been carried out have been to see if autolysis in the laboratory of foetuses removed from the mother follows the same lines as that occurring naturally. The embryos of the rat and mouse, and in one case the pig, have been used. They were incubated at 37° in boiled Ringer's solution to which toluene had been added. As a rule the embryos were left inside the membranes and the placentae were incubated with them. Autolysis rapidly begins and in some 34 hours the liver of the embryo and parts of the intestine have begun to disintegrate. The placentae soon begin to decompose

but generally the membranes remain intact until autolysis has become fairly advanced. In about 7–8 days' time the foetuses have been reduced practically to skeletons and if the medium is renewed and autolysis continued all the tissues disappear except the skeletons.

If the solution in which autolysis is proceeding is examined chemically after about a week's incubation at 37° amino-acids and peptones can be detected.

True enzyme actions are usually greatly inhibited or stopped by the addition of cyanide to the medium in which they are acting.

Foetuses from the same litter were taken (age ten days) and some were incubated at 37° in sterile Ringer-Locke solution only, while others were incubated in Ringer-Locke solution to which about 10 % KCN was added. Autolysis was allowed to proceed for a month and at the end of that time it was found that the foetuses in the Ringer and KCN solution were still intact, even to the membranes and placentae, while those in the normal Ringer were completely disintegrated.

In another experiment a partially absorbed pig embryo (length 12 cm., normal in litter 20 cm.) was taken and autolysis continued in sterile Ringer-Locke for six weeks. At the end of that time the embryo had become much more disintegrated, although complete disintegration had not occurred, probably owing to the products of the autolysis inhibiting further enzyme action.

Further experiments have been carried out in which the livers were removed from pig embryos (75 mm.) and incubated separately, the rest of the tissues being incubated at the same time as the livers. It was found that the liver rapidly autolysed, but while autolysis proceeded as usual in the rest of the embryos it did not go on at the same rate as in the isolated livers.

These, then, are the general data upon which it is possible to base any idea of the actual nature of the process, and the facts all point to the same conclusion, namely, that the dead foetus is rendered re-absorbable by some kind of enzyme activity.

Discussion.

The results of these experiments warrant the conclusion that dead foetuses are removed by autolysis and re-absorption by the maternal organism, especially as conditions in the uterus are ideal for autolysis, viz. sterility and optimum temperature. In the face of the fact that removal is obviously brought about by enzymic activity of some kind there is only one alternative explanation, namely, that the necessary ferments are produced by the uterine wall, an hypothesis which is fraught with very grave objections.

It will be noticed that in two particulars only is there any apparent difference between the observed facts of extra-uterine autolysis and the processes as known *in utero*. First, the placenta goes much earlier in the former case, and second, the disintegration of the foetuses proceeds much further *in utero*. These two discrepancies are easily explained. Normally, the foetal portion of the placenta is in intimate contact with maternal tissues, and may

therefore be supposed to die later than the foetus, whereas, during artificial incubation the placenta, which is known to contain proteolytic enzymes, is the most unstable part of the conceptus. With regard to the second point it is clear that the products of autolysis *in utero* are removed by absorption before any inhibitory concentration is attained. During incubation, on the other hand, the concentration of amino-acids increases to a stage where further breaking down is checked. That this is the explanation of the sub-total disintegration obtained is shown by the experiment where the medium was periodically changed, which resulted in the complete autolysis of the non-skeletal parts of the foetus.

It is clear, however, that the experimental conclusion reached, that the re-absorption is brought about purely by the activity of foetal enzymes, leads to rather dangerous grounds. It is, of course, known that the youngest embryo may be re-absorbed, but is it possible to carry the argument to the logical conclusion and postulate the presence of autolytic enzymes in very young embryos and even in the blastocyst?

The alternative explanation, that autolytic enzymes are present in the uterus ready to remove morbid conceptuses, hardly seems credible, since any such arrangement would form a permanent menace to normal embryos. In the case of older foetuses this problem does not arise, and in these the order of the disappearance makes it clear that some form of autolysis is at work. The mechanism of the removal of the younger embryos is, however, obscure, and upon this problem our experiments throw but little light. The youngest embryos used were those of the pig, about 75 mm. long, younger than anything which can be manipulated for incubation from the mouse. These embryos showed autolysis in the ordinary way, and it seems probable, therefore, that embryos, down at least to a very young age, may be removed by autolysis.

There is little doubt that the capacity for autolysis is a property of foetuses of any species of mammal, but in monotocous animals abortion, since the foetus is converted by death into a foreign body and this is the quickest way of clearing the uterus, usually sets in before autolysis has had time to develop. In polytocous animals, on the other hand, the death of only one or two foetuses does not seem to be sufficient to set up the activity which results in abortion, and as the dead foetus is thus retained autolysis and re-absorption have time to proceed. At the same time the fact that experimental re-absorption of the entire litter in certain polytocous animals may be produced, suggests that these animals have a greater tolerance for the products of autolysis than animals where the death of the product or products of gestation results in abortion.

Summary.

1. It has been shown by various authors that dead foetuses, especially in the younger stages, may be completely removed by some process of intra-uterine absorption.

2. The work recorded in this paper suggests that this process is one of

autolysis on the part of the dead foetus. It was found that dead foetuses will autolyse during sterile incubation, and if the products are removed when the concentration becomes inhibitive, all non-skeletal tissue goes into solution. The probability that foetuses are similarly rendered soluble in the uterus is supported by the fact that conditions in the uterus are ideal for autolysis.

3. The alternative explanation, that dead embryos are rendered soluble by uterine enzymes, is subject to grave objections, and is improbable.

BIBLIOGRAPHY.

Biedl, Peters, and Hofstatter (1921). *Z. Geburts. Gyn.* **84.**
Corner (1921). *Pub. Carnegie Inst. Wash.* No. 276.
—— (1923). *Amer. J. Anat.* **31.**
Fraenkel (1903). *Arch. Gyn.* **68.**
Hammond (1914). *J. Agr. Sci.* **6.**
—— (1921). *J. Agr. Sci.* **11.**
Huber (1915). *J. Morph.* **26.**
Koebner (1910). *Arch. Gyn.* **91.**
Long and Evans (1921). *Anat. Rec.* **21.**
Marshall (1922). Physiology of Reproduction, 2nd ed.
Meyer (1919). *Biol. Bull.* **36.**
Parkes (1923). *Proc. Roy. Soc. London*, B, **95.**
Robinson (1921). *Edin. Med. J.* **26.**

CIII. A COLORIMETRIC METHOD FOR THE DIRECT ESTIMATION OF AMMONIA IN URINE.

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For the estimation of ammonia in minute quantities the reagent almost universally adopted has been the well-known Nessler reagent either in its original form or as Folin's modification [Folin and Denis, 1916]. The use of Nessler's reagent in physiological work is attended by several difficulties, these being avoided, for the most part successfully, by ridding the reacting solutions of undesirable substances. This necessarily lengthens the process.

Tarugi and Lenci [1912] in describing some colour reactions stated that phenol added in excess to an amine or imine reacts with sodium hypochlorite to give a blue coloration. The reaction was first described by Berthelot [1859] being re-discovered by Cotton [1874] and then, as cited above, by Tarugi and Lenci. These last authors state that the reaction with ammonia is more delicate than Nessler's and they suggest its being used as the basis of a quantitative method.

The mechanism of the reaction they describe is as follows. The amine or imine in the presence of sodium hypochlorite reacts with phenol to give first *p*-nitrosophenol which in turn reacts with the excess of phenol to give *p*-benzoquinonoxyphenylimine which they state has a blue colour in solution.

Thomas [1912, 1913] was the first to work out a quantitative method for the estimation of ammonia based on the above reaction and later he applied his procedure to the detection of ammonia in the cerebrospinal fluid. A method similar to that of Thomas with slight differences of technique was later described by Foxwell [1916].

In attempting to apply the reaction to the estimation of ammonia in urine the chief difficulty encountered was the tendency of urea to react with the alkaline hypochlorite. The procedure of Thomas or of Foxwell is not applicable since in alkaline solution on heating urea breaks down to give among other products ammonia which then reacts and vitiates the results. If however the reaction is performed in the cold and the excess of phenol considerably increased the above reaction does not take place.

Of the other constituents of urine only amino acids give the reaction, and, under the conditions found suitable to eliminate the action of urea, of those tested only α-alanine and glycocoll give any reaction. The action of α-alanine

is negligible whilst glycocoll in solutions containing the same amount of amino nitrogen as an ammonia solution gives less than 5 % of the colour given by the ammonia solution.

Sodium hypochlorite solution. The intensity of the blue colour obtained depends on the concentration and amount of hypochlorite solution used. Since however this solution is not very stable it is found advisable to use for comparison in estimations a solution which contains a known amount of ammonia nitrogen per cc. The same hypochlorite being used for standard and unknown, its concentration may vary within limits. Under these conditions the strength may vary for the first week after preparation but thereafter it is reasonably constant for some months. Should the hypochlorite solution become very alkaline it should be rejected, as increase of alkali tends to impart to the colour generally obtained a green tint. A suitable concentration has been found by diluting one volume of the ordinary freshly-prepared commercial sodium hypochlorite solution with an equal volume of distilled water. The undiluted solution may be preserved in a cool dark place in a well-stoppered bottle.

Standard ammonium sulphate solution. A solution containing 0·1 mg. ammonia nitrogen per cc. is prepared by dissolving 0·4716 g. pure dry ammonium sulphate in one litre of distilled water. The pure salt may be prepared from the usual laboratory specimens as described by Folin and Denis [1916].

Method.

The following procedure was finally adopted: 20 cc. urine are diluted to 100 cc. with distilled water. Into a clean beaker of about 50 cc. capacity are weighed out 4·5 g. pure phenol crystals. To this are added 5 cc. of the well-mixed diluted urine and the phenol crystals allowed to be completely moistened. 20 cc. of the sodium hypochlorite solution are carefully added and the beaker agitated gently. The colour is allowed to develop for five minutes and the beaker then washed out with distilled water into a 250 cc. measuring flask containing about 100 cc. distilled water. The solution is then diluted up to the mark and mixed. 5 cc. of the standard ammonium sulphate solution are treated in a similar manner. The colour of the unknown is compared in a Duboscq colorimeter with that of the standard. From the comparison the ammonia nitrogen in the urine may be calculated. If the reading of the unknown be x when the standard is set at 20 for equal intensity of colour then

$$\frac{x}{20} = \frac{0{\cdot}5}{\text{mg. } NH_3N \text{ in 1 cc. urine}}$$

and this $\times 1000$ gives the number of mg. NH_3 nitrogen per litre of urine.

Should the amount of ammonia nitrogen be unusually small or unusually large the dilution of the colour should be arranged to give a convenient reading on the colorimeter.

To indicate the degree of accuracy of the method the results obtained on a series of urines compared with those obtained by the Folin macro-aeration method are shown below.

	Folin macro-method	New method
1. Normal urine	0·50	0·51
2. ,,	0·38	0·35
3. ,,	0·44	0·40
4. ,,	0·56	0·54
5. ,,	0·08	0·06
6. Diabetic urine	1·28	1·25
7. Nephritic urine	0·23	0·28
8. ,,	1·26	1·31
9. Cat urine	0·48	0·53
10. Dog urine	0·67	0·71

The following tests were carried out on solutions of ammonium sulphate, by a science student, Miss Small, who had no previous experience of the method:

Original solution mg. per cc.	Folin macro-method	New method
·025	·027	·024
·05	·05	·05
·06	·06	·06
·07	·07	·07
·08	·08	·08
·09	·09	·09
·15	·16	·16
·20	·23	·20
·25	·27	·25
·30	·31	·30

Summary.

A new and rapid method is described for the estimation of ammonia in urine colorimetrically.

The above work was done under the auspices of the Medical Research Council. I have to thank Professor D. Noël Paton for his advice and helpful criticism.

REFERENCES.

Berthelot (1859). *Repertoire de Chimie Appliqué*, p. 254.
Cotton (1874). *Bull. Soc. Chim.*
Folin and Denis (1916). *J. Biol. Chem.* **26**, 497.
Foxwell (1916). *Gas World*, 64.
Tarugi and Lenci (1912). *Boll. Chim. Farm.* **50**, 907.
Thomas (1912). *Bull. Soc. Chim.* **11**, 796.
—— (1913). *Bull. Soc. Chim.* **13**, 398.

CIV. THE MINERAL CONTENT OF HUMAN MILK IN NORMAL AND RACHITIC FAMILIES.

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(*Received June 13th, 1924.*)

The importance of an adequate supply of mineral matter in the diet to ensure normal growth and nutrition was demonstrated by experimental methods many years ago. The earliest workers observed that an insufficiency of minerals in the food of young animals invariably gave rise to defective calcification of the bones, and often to progressive malnutrition resulting in death. The exact nature of the pathological changes which follow an insufficient utilisation of one or other of the essential inorganic elements is still far from being understood.

The more recent work in experimental rickets has emphasised the importance, not only of the actual amount of the mineral matter of the diet, but also of the proportions of the individual salt constituents independently of any other factor. The metabolism of lime and of phosphoric acid has naturally received most attention in investigations of this nature.

In view of the fact that the rachitic changes in the bones of infants become manifest during the first year of life and can be detected often in the early months, the mineral content of human milk has, in the past, been subjected to critical examination in relation to the cause of rickets. Korenchevsky [1922] has collected and examined the data of earlier workers on this subject, and has discussed calcium starvation as a possibly important aetiological factor in the disease. According to the results of Bahrdt and Edelstein, Schabad, and other continental workers quoted by him, the milk of mothers of rachitic children is often subnormal in calcium content.

A number of specimens of human milk obtained from women of the poorer classes living in the industrial districts of Glasgow were examined to ascertain whether any definite deficiency of calcium or phosphorus was invariably associated with the onset of rickets. The milk on which infants developed rickets was compared with other specimens on which the infants remained free of the disease. In addition, a number of milks were selected for examination from women with healthy children living in country villages (Fifeshire and Renfrewshire).

Methods.

The milk samples were obtained by exhausting the breast artificially. In each case the maximum quantity was collected to lessen the risk of lack of uniformity in sampling, since the fat content of milk is considerably higher in the last portion withdrawn from the breast. Where it was found impossible to withdraw sufficient milk for analysis, specimens were collected from the same subject daily over a period of three or four days and mixed.

A series of estimations was carried out on specimens of milk collected in three successive and approximately equal quantities of 40 cc. in a number of different cases. These results (Table I) show that there was practically no variation in the mineral content with the degree of breast exhaustion. The increase of fat concentration was much less than that which has been observed in animal milks.

The estimations of fat, total solids, ash, lime and phosphoric acid were made by the usual methods (Table II).

Table I. *Examination of human milks collected in three successive portions* of 40 *cc.*

	Fat %			Total solids %			Ash %			CaO %		
No.	(1)	(2)	(3)	(1)	(2)	(3)	(1)	(2)	(3)	(1)	(2)	(3)
1.	2·84	4·97	5·28	—	—	—	—	—	—	—	—	—
2.	2·32	4·03	6·19	—	—	—	—	—	—	—	—	—
3.	4·36	5·77	7·20	14·09	15·66	16·23	0·19	0·17	0·18	—	—	—
4.	0·4	1·33	1·96	10·43	11·30	11·58	0·21	0·24	0·20	—	—	—
5.	1·12	1·4	1·48	10·75	11·24	11·54	0·23	0·25	0·21	—	—	—
6.	3·4	4·0	5·3	12·41	13·2	14·7	0·23	0·22	0·24	0·055	0·053	0·053
7.	2·17	2·93	3·31	11·48	12·65	12·77	0·18	0·20	0·19	0·033	0·031	0·031
8.	1·19	2·58	3·12	10·72	12·02	13·38	0·17	0·17	0·16	0·033	0·032	0·033

Table II. *Mineral content of human milk in rachitic and non-rachitic families (Industrial Class—Town Dwellers).*

Group A. Non-rachitic.

No.	Age of mother (years)	Period of lactation	No. of children	% Fat	Ash	CaO	P_2O_5
1	20	3 weeks	2	1·58	0·18	0·048	0·038
2.	25	4 ,,	1	3·16	0·24	0·04	0·036
3.	—	5 ,,	1	1·74	0·18	0·04	0·04
4.	35	7 ,,	5	2·68	0·20	0·044	0·033
5.	29	3 months	4	0·6	0·17	0·05	0·036
6.	23	4 ,,	2	2·79	0·20	0·055	0·048
7.	27	4 ,,	1	2·34	0·23	0·05	0·03
8.	20	6 ,,	3	1·96	0·23	0·06	0·05
9.	25	6 ,,	2	1·72	0·21	0·045	0·039
10.	36	6 ,,	2	3·28	0·15	0·044	—
11.	25	7 ,,	3	1·8	0·20	0·04	0·04
12.	24	13 ,,	3	3·23	0·16	0·048	0·04
			Average	2·24	0·196	0·047	0·04

Group B. Rachitic.

No.	Age of mother (years)	Period of lactation	No. of children	% Fat	Ash	CaO	P_2O_5
1.	36	3 weeks	4	2·08	0·24	0·048	0·035
2.	28	5 „	5	3·21	—	0·06	0·06
3.	20	2 months	1	3·74	0·16	0·04	0·04
4.	27	2 „	3	2·35	0·19	0·05	0·05
5.	39	3 „	8	0·3	0·16	0·04	0·03
6.	30	3 „	4	2·51	0·20	0·05	0·032
7.	42	3 „	11	5·15	—	0·05	0·04
8.	28	4 „	5	1·6	0·22	0·048	0·023
9.	31	4 „	6	3·61	0·17	0·05	0·045
10.	29	4 „	9	3·53	—	0·05	—
11.	30	7 „	5	3·59	0·25	0·056	0·045
12.	23	8 „	2	3·91	—	0·04	0·036
13.	27	1 year	3	0·92	—	0·05	0·05
14.	30	1 „	3	2·33	0·20	0·044	0·024
15.	32	1 „	4	3·99	0·16	0·034	0·037
			Average	2·85	0·195	0·047	0·039

Group C. Milk of Women living in Country Villages

No.	Age of mother (years)	Period of lactation	No. of children	% Fat	Ash	CaO	P_2O_5
1.	44	5 days	10	2·07	0·22	0·035	0·05
2.	24	10 „	2	3·19	0·17	0·045	0·045
3.	36	10 „	2	2·3	0·17	0·033	0·05
4.	18	12 „	1	4·32	0·23	0·054	0·056
5.	27	12 „	3	2·8	0·19	0·032	0·039
6.	24	17 „	2	2·2	0·20	0·04	0·046
7.	25	4 weeks	2	3·66	0·18	0·035	0·037
8.	26	5 „	3	4·02	0·23	0·04	0·04
9.	34	6 „	11	3·67	0·17	0·049	0·05
10.	43	2 months	11	2·15	—	0·05	0·04
11.	27	3 „	3	5·03	—	0·042	0·035
12.	23	6 „	1	2·66	0·20	0·047	0·043
			Average	3·17	0·196	0·042	0·044

Results.

No appreciable differences in the mineral content of the milks of the non-rachitic and rachitic groups were observed, the average figures obtained being nearly identical. In both groups there is a considerable range of variation of lime, phosphoric acid and fat, but in each series the CaO of most of the specimens lies within the limits 0·04–0·05 %. In the rachitic group only one specimen was below 0·04 %. Several milks in both groups contained ap preciably less phosphoric acid than lime. These were probably abnormal in this respect, since a slight excess of phosphoric acid over the calcium equivalen necessary for bone formation is required for the growth of the soft tissues. That this excess is normally supplied in milk is shown by the following examples, taken from the compilation on phosphorus metabolism by Forbes and Keith [1914].

	Cow	Sheep	Goat	Pig	Dog	Mare
CaO %	0·168	0·245	0·197	0·257	0·44	0·123
P_2O_5 %	0·215	0·292	0·284	0·317	0·48	0·130
Author	Sherman		Abderhalden			Bunge

A number of specimens in both groups were very deficient in fat, a feature not uncommon in the milk of women among the poorer industrial classes. In a study of the calorific value of human milk in relation to marasmus made by the late Major H. S. Hutchison, several of the specimens obtained from women attending a children's dispensary were almost transparent.

In Group C, the lime content is very slightly less than in the preceding groups, four of the specimens containing less than 0·04 % CaO, but the phosphoric acid content is distinctly higher than the lime. The milks of the women living in the country were therefore relatively richer in phosphorus than those of women living under poor conditions in the town.

Korenchevsky [1922] has drawn attention to an observation of Pfeiffer on the ash of human milk, that the percentage of phosphoric acid of the total ash is subnormal in the milk of mothers of rachitic children. This observation is based on the results obtained from the examination of only three "rachitic" milks, and in one of these the percentage of phosphoric acid quoted (24·99 %) is higher than the normal average (24·7 %) determined by Pfeiffer.

In Groups A and B, the ash contained 20 % of phosphoric acid, and the latter was equal to or less than the lime in all the specimens. In Group C, 22·4 % of phosphoric acid was present in the ash, while, with two exceptions, it was equal to or greater than the lime. No milks abnormally poor in fat were obtained from country sources. One milk was extremely high in mineral content. Three specimens collected on different days gave the following results:

Fat	Ash	CaO	P_2O_5
4·52	0·41	0·096	0·089
3·96	—	0·084	0·076
4·06	0·33	0·088	0·076

De Buys and Von Meysenbug [1924] have recently published the results of calcium determinations in a large series of breast milks. In 55 cases in which the babies developed rickets, they found the calcium percentages varying from 18·4 mg. (0·025 % CaO) to 39·6 mg. (0·055 % CaO). The average for 19 normal milks was 32·6 mg. (0·045 % CaO). In some of the most severe cases of rickets the calcium content of the milk was well within the normal range. On the other hand, very low extremes in the calcium content of the rachitic group were recorded as compared with the normal series. These observers corroborate the findings of Burhaus and Smith [1923], that the average calcium content of the milk in white races (30·79 mg. Ca %) is higher than that of coloured women (25·41 mg. Ca %), an interesting observation in view of the fact that rickets is more prone to develop in negro children.

The results presented here confirm the conclusions of Schabad [1911] that infantile rickets can occur when the calcium content of the breast milk is normal or even above normal, so that a deficiency of calcium in the milk cannot be the only cause of rickets.

I am indebted to Prof. D. Noël Paton for helpful advice and criticism in this work, the expenses of which were defrayed by a grant from the Medical Research Council.

SUMMARY.

1. The mineral content of 27 specimens of breast milk of women living under poor conditions in the industrial quarters of Glasgow was determined.

2. No appreciable differences were found in the composition of the milks, whether the infants developed rickets or did not. The fat content of several specimens of both groups was abnormally low.

3. Twelve specimens of milk from healthy women living in country villages were examined for purposes of comparison. In these the ratio of phosphoric acid to lime was slightly higher than in the milk of women living in a poor environment.

4. Schabad's conclusion that infantile rickets may develop on breast milk with a high calcium content is confirmed.

REFERENCES.

Burhaus and Smith (1923). *Amer. J. Dis. Child.* **26**, 303.
De Buys and Von Meysenbug (1924). *Amer. J. Dis. Child.* **27**, 438.
Forbes and Keith (1914). *Ohio Agric. Exp. Station Bull.* No. 5. Technical Series.
Korenchevsky (1922). *Med. Res. Council Spec. Rep. Series*, No. 71.
Schabad (1911). *Jahrb. Kinderheilk.* **74**, 511.

CV. AMMONIA PRODUCTION BY ANIMAL TISSUES IN VITRO.

I. THE USE OF MIXED TISSUE EXTRACTS.

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(*Received June 14th, 1924.*)

One of the earliest theories of protein metabolism was based on the observation by Nencki and colleagues [1896, 2] that the portal blood contained considerably more ammonia than the systemic blood. To explain this fact, the theory was developed that the absorbed amino acids were immediately deaminised—probably in the intestinal wall. The work of Cohnheim [1909, 1912] on the deamination of amino acids by fish intestines in Ringer's solution, and the observation of Horodynski, Salaskin and Zaleski [1902] that even in starvation the ammonia content of the portal blood was greater than that of the systemic blood, considerably strengthened the theory of deamination by the intestine. The earlier weaknesses of the theory may be stated as follows:

(1) Cohnheim was not successful in demonstrating the deamination of amino acids by mammalian intestines (those of dogs and cats).

(2) The work of Horodynski, Salaskin and Zaleski [1902] mentioned above, is contradicted by Nencki's original observation.

The work of Folin and Denis [1912, 1, 2] gave to Nencki's observations a different interpretation than that of deamination by the intestinal wall. They found that the ammonia of the portal blood of cats came principally from the mesenteric vein of the large intestine and therefore concluded that the portal ammonia entered by absorption from the ammonia-rich faeces in the large intestine. Neither the amino acids of a pancreatic autolysate, nor glycine, nor asparagine were deaminised by the small intestine when injected into ligatured loops of that organ. Folin and Denis assumed that the blood rapidly transported the absorbed and undeaminised amino acids from the intestine to the tissues. Van Slyke and Meyer [1913] confirmed these results. By the use of Van Slyke's newly-developed method for the estimation of amino acid nitrogen, they were able to show [1913] that half an hour after the injection of totally hydrolysed caseinogen, 5 % of the amino nitrogen was still in the circulation, 11 % had been excreted in the urine, while the increased amino nitrogen content of the various tissues accounted for almost all of the remainder.

We are now concerned with the next step in amino acid catabolism. It has long been known that the ingestion of proteins is followed by an in-

creased excretion of urea. The early demonstration of v. Schroeder [1882] that the liver is able to synthesise urea from the ammonium salts of organic acids has been amply confirmed by much subsequent work. The Eck fistula experiments of Nencki and colleagues [1896, 1] demonstrated that in dogs, functional blocking of the liver decreased the urea excretion and increased the ammonia excretion. The normal ability of the animal to form urea from carbaminic acid (introduced *per os*) also disappeared. "Die Leber ist also der treue Wächter des Organismus, der die von dem Verdauungscanal kommende, für die anderen Organe giftigen Substanzen, in ungiftige verwandelt" [Nencki et al., 1896, 2].

The question then arose as to whether the liver could form urea from amino acids without the intermediate stage of ammonia liberation by deamination.

The suggestion had long been made that urea might be formed from amino acids by the intermediate formation of uramino acids [Salkowski, 1880].

$$R \cdot CH(COOH) \cdot NH_2 + CO_2 + NH_3 \longrightarrow R \cdot CH(COOH) \cdot NH \cdot CO \cdot NH_2$$
$$\longrightarrow R \cdot CH(COOH) \cdot OH + CO(NH_2)_2.$$

Recent work [Lewis, 1912; Rohde, 1918; Lewis and Root, 1921], however, has failed to demonstrate that such a mechanism exists *in vivo*. One might therefore conclude that in the liver, at least, urea may be formed from ammonium salts of organic acids, but that its direct formation from amino nitrogen is still a matter of doubt.

As regards the locus of ammonia formation arguments have already been advanced which render dubious the older theory that the intestinal mucosa is the seat of this change. Nash and Benedict [1921, 1922] have advanced the view that ammonia formation is a function of the kidney. They found the ammonia content of the blood drawn from the renal veins of anaesthetised dogs to be higher than that of arterial blood and of the blood from the inferior vena cava. This work was repeated by Henriques [1923], but the observations of Nash and Benedict were not confirmed. The valuable experiments of Neubauer and Fisher [1910] on liver perfusion demonstrated that this organ (the liver) was probably responsible for *in vivo* deamination. Subsequent workers [Fiske and Karsner, 1913] have had occasion to dispute the alleged deaminising power of the liver. Folin and Denis [1912, 1] state: "We soon discovered that while the liver does almost wholly abstract the ammonia from the portal blood and probably converts it into urea, it does not 'deaminise' the amino acids." These conclusions with fresh experimental evidence were restated at a recent date [Folin and Berglund, 1922]. Levene and Meyer [1913, 1, 2] were unsuccessful in demonstrating the deamination of various amino acids by the kidney, liver, or polymorphonuclear leucocytes. Similar results were reported by Griesbach and Oppenheimer [1913]. Levene and Meyer [1913, 2] conclude as follows: "These observations make it necessary to repeat all the older work on the deaminising action of tissues."

While investigating the amide nitrogen of animal proteins, the writer was attracted to the problem. It appeared desirable to make an extensive study of the deaminising activity of many tissue preparations on different substrates.

The use of mixed tissue extracts was suggested by Professor Hopkins, with the idea that a given organ may elaborate a hormone which stimulates deamination in other parts of the body. In addition to the estimation of free ammonia, the estimation of the urea production was also obviously desirable. The fact that all animal proteins contain amide nitrogen, and that these amides must have been synthesised by the animal body, suggested the estimation of amide nitrogen. The observation recorded in a previous paper [Luck, 1924] that a portion of the amide nitrogen of caseinogen was readily split off as ammonia by various tissues of the body, and the older observations on the ease of hydrolysis of amides by tissue extracts, suggested that one mechanism of ammonia production might be the synthesis of amides by certain organs and deamidation by others.

During the course of the first experiment certain observations of Nencki and of Van Slyke led to the use of the gastric mucosa in Exps. 2 and 3.

Nencki and his colleagues [1896, 2] by improved methods of analysis found that the gastric mucosa of dogs contained considerably more free ammonia than any other tissue investigated and ascribed this to a decomposition of proteins in the mucosa during the formation of the gastric juice.

Seventeen years later, Van Slyke [1913] observed that the stomach contained less amino acid nitrogen than any other tissue examined.

This feature of highest ammonia content and lowest amino nitrogen content suggested that the gastric mucosa might play a prominent part in ammonia production. That this is actually the case will be seen from the experimental results which follow.

Experimental.

The following technique was adopted. In the preparation of tissue extracts, the excised tissue was rinsed in cold, running, water, then minced and ground with washed sand, water and a small quantity of toluene until a fine suspension was obtained. Sufficient water was added to adjust the concentration of tissue material to approximately 10 %. In the case of the pituitary body, lymph glands, and submaxillary glands, the insufficiency of tissue necessitated a lower concentration. The bile and lymph were used without dilution. In Exp. 1 an animal was used which had fasted for 18 hours so that the lymph was practically free from fat and red cells. 55 cc. were collected in 4 hours and run into 10 cc. of 1 % potassium oxalate. The animal was killed by exsanguination from the external carotid. A portion of the blood was defibrinated as collected. The remainder was oxalated and centrifuged. The formed elements were twice washed with isotonic sodium chloride solution, and then laked by the addition of an equal volume of water.

In the preparation of the red bone marrow, the difficulty of separating the marrow from the bone was encountered. It was found convenient to use the ribs. These were carefully scraped free of adherent tissue, quartered by longitudinal section, cut into centimetre lengths, and used as such.

Three different substrates were investigated:

1. *Totally hydrolysed gelatin.* 280 g. of gelatin were hydrolysed by boiling with 1000 cc. of water and 135 cc. of concentrated sulphuric acid for 22 hours at atmospheric pressure. The sulphuric acid was removed with baryta, the filtrate adjusted to p_H 7·6 with soda, and diluted to an amino nitrogen concentration of 8·85 mg. per cc.

2. *Trypsin-hydrolysed caseinogen.* Caseinogen was hydrolysed in 15 % solution by an aqueous extract of the pancreas at p_H 7·8. The digestion was allowed to continue for 8 weeks with the weekly addition of fresh pancreas extract. The digest was then slightly acidified with acetic acid, filtered, boiled, and adjusted with soda to p_H 7·6. Water was added until the amino nitrogen concentration was 8·85 mg. per cc.

3. *Trypsin-stable amide residue from caseinogen.* This was prepared by alcoholic precipitation and extraction as described in a previous paper [Luck, 1924]. The concentration of amino nitrogen was adjusted to 8·85 mg. per cc. and the hydrogen ion concentration to p_H 7·6.

In the text, these are designated as Substrate 1, 2 and 3 respectively. Substrate 1 contains no amide nitrogen. Substrate 2 contains both amide and amino nitrogen. Substrate 3 contains a relatively large amount of trypsin-stable amide nitrogen, while the amino nitrogen is limited to that of two amino acids, glutaminic acid and lysine.

The detailed procedure was as follows: 4 cc. portions of the substrate were placed in test tubes ($6'' \times \frac{5}{8}''$)—previously sterilised by steaming for 3 hours. 4 cc. of the tissue preparation were added. In those cases where mixed tissue suspensions were used, only 2 cc. of each were employed. To each tube were then added 1·5 cc. of water, 0·05 cc. of 5 % calcium chloride and 0·5 cc. of toluene. The tubes were thoroughly shaken, stoppered tightly with sterilised corks, and kept at 40°. While careful control of the hydrogen ion concentration throughout the period of incubation would have been a matter of great difficulty, the initial p_H of the substrate (7·6) was such that the final p_H was never found to be lower than 7·0. After five days the tubes were removed. Twelve tubes were put aside for immediate analysis, and the remainder placed in a refrigerator at 0–1°.

The contents of each daily lot of twelve were acidified with a single drop of glacial acetic acid and boiled for 15 minutes in a water-bath. The volume of each was then rapidly adjusted to 10 cc. in volumetric flasks and filtered. Of the filtrate, 3 cc. were used for the estimation of ammonia, 3 cc. for urea, and 2 cc. for amide nitrogen.

Ammonia was estimated by Van Slyke and Cullen's [1914] modification of Folin's aeration method. By the use of three pumps, six estimations could be made in 50 minutes.

In the estimation of urea, the 3 cc. samples were placed directly in the aeration tubes. Three drops of phenol red solution were added, and 0·5*N* soda added by drops until the indicator changed colour. 2 cc. of phosphate buffer

(p_H 7·4), 0·3 g. of soya bean meal, and four drops of caprylic alcohol were added to each tube. They were kept for 2 hours at 40° and the ammonia then estimated as above.

For the estimation of amide nitrogen, the 2 cc. samples were placed in 6″ combustion tubes with 3 cc. of water and 0·5 cc. of concentrated sulphuric acid. In the first experiment the tubes were then heated for 3 hours in an oil bath at 105–110°. It was soon found to be more satisfactory to heat for 2 hours in an autoclave at 5–10 lbs. pressure. Under such conditions urea is only slightly destroyed (Table VIII). The tubes were cooled, and the acid removed by the addition to each of 5 cc. of a warm baryta solution containing 2·95 g. of crystalline baryta per 5 cc. A few drops of bromocresol purple were added as an aid in this approximate neutralisation. The tubes were cooled and the ammonia content estimated as above without transfer of the contents.

The deaminising activity of the following tissues and fluids was investigated:

Muscle, *A*; liver, *B*; mucosa of the small intestine, *C*; adrenals, *D*; thyroid gland, *E*; spleen, *G*; pituitary body, *H*; parotid glands, *J*; submaxillary glands, *K*; gastric mucosa, *M*; pancreas, *N*; mesentery, *O*; bile, *P*; kidney, *Q*; ovaries, *R*; brain, *S*; heart, *T*; lymph, *U*; lymph glands, *V*; whole blood, *W*; defibrinated blood, *X*; formed elements, *Y*; red bone marrow, *Z*. The mixed diaphragm and pectoral muscles were used in the preparation of *A*. The letters following the name of the tissue or fluid have been used in the text as an abbreviated means of designation.

Exp. 1. The dog, a female of 10 kilos., was injected subcutaneously with 2·5 cc. of 2 % morphia. After one hour the animal was anaesthetised with alcohol and ether. The thoracic duct was approached through the subclavian triangle and the lymph collected over a period of 3 hours. It was then bled from the external carotid and the tissues and glands rapidly excised. Owing to the large amount of material that had to be prepared, a number of the organs were kept overnight at 0–1°. The kidneys, liver, intestine, stomach, bile, lymph, and blood were prepared and tubed at once. The results are expressed in Tables I–V which follow.

Table I. *Blanks with water.*

	cc. *N*/10 nitrogen per 3 cc.				cc. *N*/10 nitrogen per 3 cc.		
	Ammonia N	Amide N (nett)	Urea N (nett)		Ammonia N	Amide N (nett)	Urea N (nett)
Substrate 1	0·20	0·00	0·05	*O*	0·02	0·01	0·03
,, 2	0·55	0·35	0·05	*P*	0·02	0·00	0·05
A	0·02	0·01	0·01	*Q*	0·10	0·05	0·05
B	0·07	0·01	0·06	*R*	0·02	0·00	0·03
C	0·03	0·05	0·10	*S*	0·00	0·02	0·05
D	0·05	0·05	0·05	*T*	0·05	0·00	0·00
E	0·05	0·05	0·03	*U*	0·05	—	0·10
G	0·07	0·08	0·08	*V*	0·00	0·02	0·15
H	0·02	0·01	0·03	*W*	0·05	—	0·13
J	0·03	0·05	0·04	*X*	0·05	—	—
K	0·03	0·00	0·07	*Y*	0·10	—	0·00
M	0·13	0·07	0·05	*Z*	0·10	—	0·00
N	0·15	0·15	0·09				

In Tables II–V the blanks given above have been subtracted from the analytical figures in order to indicate the degree, if any, of ammonia liberation.

Table II. *Muscle mixtures.*

	Substrate 1 cc. *N*/10 nitrogen per 3 cc.			Substrate 2 cc. *N*/10 nitrogen per 3 cc.		
	Ammonia N (nett)	Amide N (nett)	Urea N (nett)	Ammonia N (nett)	Amide N (nett)	Urea N (nett)
A	−0·05	0·07	0·05	−0·04	0·32	0·02
A B	0·07	−0·02	0·95	0·40	−0·02	0·35
A C	0·03	0·02	0·56	0·20	0·20	0·15
A D	0·03	0·08	0·20	0·11	0·22	−0·03
A E	−0·03	−0·04	0·03	0·08	0·20	0·02
A G	0·04	−0·04	0·14	0·03	0·30	0·02
A H	0·00	−0·05	0·07	0·03	0·31	−0·03
A J	0·00	0·01	0·00	0·04	0·29	−0·01
A K	0·03	0·25	0·05	0·03	0·40	0·05
A N	0·03	2·28	−0·03	0·10	1·28	0·05
A O	0·05	−0·05	−0·05	0·08	0·28	0·00
A P	0·03	−0·04	0·02	0·05	0·68	0·00
A Q	0·05	−0·05	0·25	0·20	0·05	0·15
A R	0·00	0·00	0·00	0·05	1·13	−0·05
A S	−0·05	0·00	0·10	0·00	0·28	0·10
A U	0·10	−0·02	0·05	0·00	0·31	0·05
A X	−0·05	−0·05	0·13	−0·05	—	0·05
A Y	0·05	−0·04	0·05	0·15	0·06	0·00
A Z	0·45	0·05	−0·05	0·55	0·03	0·10

Table III. *Liver mixtures.*

	Substrate 1 cc. *N*/10 nitrogen per 3 cc.			Substrate 2 cc. *N*/10 nitrogen per 3 cc.		
	Ammonia N (nett)	Amide N (nett)	Urea N (nett)	Ammonia N (nett)	Amide N (nett)	Urea N (nett)
B	0·10	−0·05	0·95	0·50	−0·10	0·40
B A	0·07	−0·02	0·95	0·40	−0·02	0·35
B C	0·12	−0·02	1·05	0·55	0·38	0·35
B D	0·07	0·28	1·05	0·45	0·06	0·35
B E	0·07	−0·02	1·07	0·50	0·40	0·30
B G	0·10	0·00	1·07	0·51	0·75	0·37
B H	0·10	−0·05	1·05	0·58	0·60	0·25
B J	0·10	0·18	1·00	0·55	0·33	0·25
B K	0·09	−0·02	0·96	0·40	0·53	0·30
B N	0·10	0·03	1·27	0·35	0·30	0·40
B O	0·00	−0·03	0·67	0·45	0·43	0·30
B P	−0·03	—	1·08	0·25	0·56	0·30
B Q	0·02	−0·05	1·15	0·65	0·31	0·50
B R	0·10	0·00	1·07	0·50	0·61	0·25
B S	—	—	—	0·40	−0·07	0·28
B U	0·14	−0·04	0·93	0·43	−0·10	0·32
B X	0·07	—	0·87	0·25	0·18	0·37
B Y	0·22	0·00	0·85	0·70	0·30	0·45
B Z	0·62	−0·05	0·75	0·75	0·21	0·35

Table IV. *Intestinal mucosa mixtures.*

	Substrate 1 cc. $N/10$ nitrogen per 3 cc.			Substrate 2 cc. $N/10$ nitrogen per 3 cc.		
	Ammonia N (nett)	Amide N (nett)	Urea N (nett)	Ammonia N (nett)	Amide N (nett)	Urea N (nett)
C	0·04	−0·03	0·83	0·15	0·23	0·27
C A	0·03	0·02	0·56	0·20	0·20	0·15
C B	0·12	−0·02	1·05	0·55	0·38	0·35
C D	0·07	0·06	0·60	0·25	0·41	0·07
C E	0·04	−0·04	0·31	0·18	0·12	0·10
C G	0·02	−0·04	0·70	0·20	0·07	0·13
C H	0·02	−0·05	0·48	0·10	0·18	0·15
C J	0·00	−0·03	0·40	0·14	0·40	0·13
C K	0·00	0·01	0·45	0·12	0·21	0·26
C N	0·01	0·00	0·06	0·16	0·14	0·02
C O	−0·03	0·01	0·60	0·03	0·18	0·17
C P	0·01	−0·06	—	0·20	0·38	0·00
C Q	0·00	0·00	0·87	0·20	−0·04	0·42
C R	−0·03	0·00	0·47	0·23	0·05	0·07
C S	0·02	−0·05	0·45	0·10	0·15	0·20
C U	0·02	−0·06	0·63	0·20	0·38	0·10
C X	−0·03	0·05	—	0·02	0·11	0·08
C Y	0·02	−0·06	—	0·25	0·33	0·10
C Z	0·02	0·05	0·70	0·90	−0·15	0·10

Table V. *Single tissues and fluids.*

	Substrate 1 cc. $N/10$ nitrogen per 3 cc.			Substrate 2 cc. $N/10$ nitrogen per 3 cc.		
	Ammonia N (nett)	Amide N (nett)	Urea N (nett)	Ammonia N (nett)	Amide N (nett)	Urea N (nett)
A	−0·05	0·07	0·05	−0·04	0·32	0·02
B	0·10	−0·05	0·95	0·50	−0·10	0·40
C	0·04	−0·03	0·83	0·15	0·23	0·27
D	0·08	−0·05	0·10	0·08	0·23	0·00
E	0·03	0·65	0·02	0·03	0·25	0·05
G	0·03	−0·05	0·14	0·05	0·61	0·00
H	0·01	0·01	0·02	0·03	0·21	—
J	−0·03	0·01	0·03	0·05	0·43	0·55
K	0·03	−0·04	—	—	—	—
M	0·11	−0·02	0·14	0·23	0·70	−0·10
N	0·15	1·40	0·03	0·20	1·06	0·25
O	0·02	−0·02	—	—	—	—
P	−0·05	0·13	0·07	−0·05	0·48	0·05
Q	0·05	−0·07	0·67	0·40	0·02	0·30
R	−0·03	0·00	−0·02	−0·05	0·33	0·10
S	0·03	−0·05	0·09	0·10	0·30	−0·05
T	0·00	−0·10	0·00	0·20	0·28	−0·08
U	0·05	−0·03	0·03	0·05	0·25	−0·05
V	0·00	0·20	−0·05	0·05	0·78	0·02
W	0·02	—	0·08	−0·10	—	0·10
X	0·00	0·01	—	−0·05	—	0·15
Y	0·10	−0·05	—	0·20	0·23	0·05
Z	0·65	−0·07	0·05	0·65	0·31	0·03

CONCLUSIONS. *Exp.* 1. (1) Free ammonia from α-amino acids was liberated by the red bone marrow only, except in combination with the intestinal mucosa where no deamination was observed. The fact that in all the tubes but this one the ribs projected above the surface of the liquid, and might therefore become septic through lack of toluene, would suggest bacterial deamination. This impression is heightened by subsequent failure to obtain deamination by the red bone marrow (Exp. 3).

(2) Free ammonia was liberated from trypsin-digested caseinogen by the liver, kidney, and very slightly by the intestinal mucosa. The failure of these organs to deaminise totally hydrolysed gelatin, as well as the fact that these organs liberate ammonia from Substrate 3, but not from acid-hydrolysed Substrate 3 [Luck, 1924], suggests that in this instance the mechanism is one of deamidation and not deamination.

(3) If this is so, the sum of free ammonia nitrogen and amide nitrogen in the experiments with Substrate 2 should be 0·35, viz. the amide nitrogen content of Substrate 2. Owing to the errors introduced by blanks and the number of manipulations required, this process of summation can only be expected to yield approximate results. However, Tables II, IV and V indicate by this method that the process of ammonia formation is one of deamidation. In Table II, Substrate 2, the sum of ammonia nitrogen and amide nitrogen is much greater in most cases than 0·35. This indicates either

(*a*) the liberation of ammonia from α-amino groups,

or (*b*) the synthesis of a material which by mild acid hydrolysis liberates ammonia.

The improbability of (*a*) has already been mentioned. That process (*b*) is more likely will appear from the results of Exps. 2 and 3 which follow.

(4) Urea was formed by preparations of the liver, intestinal mucosa and kidney.

It is probable that the urea has been formed from arginine by the action of arginase.

Exp. 2. A fresh supply of Substrate 2 was required. After filtration and boiling, the solution was made slightly alkaline with calcium hydroxide and distilled *in vacuo* for the removal of free ammonia. It was then filtered and adjusted to p_H 7·6 and an amino nitrogen concentration of 10·0 mg. per cc.

5 cc. of each substrate were used instead of 4 cc. as in Exp. 1. No prolonged period of anaesthesia as in Exp. 1 preceded the excision of the tissues. Duplicate mixtures of tissue and substrate were used throughout. 4 cc. of the filtrates were used in the ammonia estimations, and 5 cc. for the estimation of amide nitrogen. Urea was not estimated.

The dog, a healthy female of 10 kilos., was killed by exsanguination after 12 hours of fasting. The tissues were rapidly excised and prepared as previously described. The results are arranged in Tables VI, VII.

Table VI. *Single tissues and fluids.*

	Substrate 1 cc. $N/10$ nitrogen per 5 cc.		Substrate 2 cc. $N/10$ nitrogen per 5 cc.		Substrate 3 cc. $N/10$ nitrogen per 5 cc.	
	Ammonia N (nett)	Amide N (nett)	Ammonia N (nett)	Amide N (nett)	Ammonia N (nett)	Amide N (nett)
B	0·29	0·63	0·52	1·00	1·36	0·94
B	0·25	0·64	0·55	0·90	1·32	0·95
C	0·07	0·02	0·12	0·68	0·21	1·51
C	0·00	0·12	0·12	0·58	0·20	1·42
G	0·00	0·09	0·02	0·73	0·07	1·50
G	0·04	0·08	0·08	0·70	0·07	1·58
M	0·10	0·00	0·37	0·53	1·01	0·89
M	0·07	0·03	0·25	0·45	1·02	1·00
N	0·04	−0·03	0·03	0·42	0·07	1·35
N	0·04	−0·02	0·00	0·70	0·03	1·40
P	0·04	0·08	−0·04	0·83	−0·08	1·75
P	−0·02	0·10	0·02	0·70	−0·08	1·83
Q	0·11	0·03	0·11	0·71	0·22	1·55
Q	0·07	0·13	0·17	0·62	0·27	1·55
Water	0·00	0·06	0·00	0·70	0·00	1·62
,,	0·00	0·00	0·00	0·73	0·00	1·60

Table VII. *Mixed tissues and fluids.*

	Substrate 1 cc. $N/10$ nitrogen per 5 cc.		Substrate 2 cc. $N/10$ nitrogen per 5 cc.		Substrate 3 cc. $N/10$ nitrogen per 5 cc.	
	Ammonia N (nett)	Amide N (nett)	Ammonia N (nett)	Amide N (nett)	Ammonia N (nett)	Amide N (nett)
B C	0·28	0·94	0·50	0·73	1·07	1·20
B C	0·19	0·93	0·50	0·85	1·11	1·11
B G	0·29	0·88	0·38	1·02	1·01	1·36
B G	0·25	0·89	0·39	0·94	0·92	1·60
B M	2·29	−0·12	1·13	0·70	2·32	0·73
B M	2·44	−0·12	1·25	0·55	2·32	0·70
B N	0·20	0·92	0·09	1·19	0·76	1·51
B N	0·19	0·80	0·15	1·00	0·73	1·47
B P	0·07	0·63	0·19	0·76	0·60	1·17
B P	0·07	0·67	0·21	0·84	0·67	1·35
B Q	0·19	0·90	0·56	0·64	1·15	1·02
B Q	0·25	0·74	0·53	0·77	1·07	1·33
C M	0·54	−0·05	0·34	0·31	1·17	0·98
C M	0·48	−0·04	0·38	0·25	1·02	1·10
N M	0·04	0·08	0·03	0·78	0·10	1·62
N M	0·04	0·10	0·00	0·83	0·11	1·54
N P	0·02	−0·02	−0·01	0·66	0·05	1·68
N P	0·04	0·06	0·00	0·70	0·05	1·55
N C	0·04	−0·07	0·03	0·77	0·20	1·50

Exp. 3. A male dog of 8 kilos. which had fasted for 12 hours was used in this experiment. The substrate and details of procedure were the same as those of Exp. 2. The results are recorded in Table VIII.

Table VIII.

	Water cc. $N/10$ nitrogen per 5 cc.		Substrate 1 cc. $N/10$ nitrogen per 5 cc.		Substrate 2 cc. $N/10$ nitrogen per 5 cc.	
	Ammonia N (nett)	Amide N (nett)	Ammonia N (nett)	Amide N (nett)	Ammonia N (nett)	Amide N (nett)
B	—	—	0·27	0·55	0·63	0·70
B	—	—	0·32	0·60	0·67	0·66
M	—	—	0·32	0·02	0·40	0·45
M	—	—	0·32	−0·03	0·42	0·40
N	—	—	0·23	0·11	0·25	0·63
N	—	—	0·25	0·12	—	0·58
Y	—	—	0·23	—	—	—
Y	—	—	0·17	—	—	—
Z	—	—	0·04	0·18	—	—
Z	—	—	0·04	0·08	0·06	—
B M	0·23	−0·03	2·19	−0·17	1·25	0·60
B M	0·23	0·00	2·29	−0·17	1·25	0·50
B N	0·19	0·11	0·34	0·80	0·38	1·02
B N	0·23	0·08	0·36	0·86	0·34	0·99
N M	0·19	0·01	0·29	−0·02	0·21	0·64
N M	0·19	0·06	0·32	−0·02	0·19	0·71

	Ammonia N	Amide N	Urea N
Urea	0·00	0·18	2·00
„	0·01	0·12	2·05

Conclusions. *Exps.* 2 *and* 3. 1. Marked ammonia production was observed in mixtures of the gastric mucosa and liver preparations. Quantitative agreement of duplicate sets from a single animal and of preparations from both animals was also observed.

2. No appreciable deamination by either the gastric mucosa or liver, or other single tissue preparation could be demonstrated.

3. Definite deamidation was effected by the liver, gastric mucosa, kidney, intestinal mucosa, and most liver mixtures. (See Substrates 2 and 3, Tables VI–VIII.)

4. The synthesis by the liver and liver mixtures of an amide-like body (a substance yielding ammonia after mild acid hydrolysis) was also demonstrated. It is possible that the ammonia so liberated might have been formed by the destruction of urea, though a similar concentration of urea in 20 % sulphuric acid and under identical conditions of hydrolysis was only slightly hydrolysed.

5. Of all the liver mixtures investigated with totally hydrolysed gelatin, no residual amide nitrogen could be found in the gastric mucosa-liver experiments (Substrate 1, Tables VII, VIII).

To Professor F. G. Hopkins the writer is indebted for advice and helpful criticism. Dr L. E. Shore very kindly performed the thoracic duct operation, while the assistance of Dr C. S. Hicks in dissection is gratefully acknowledged. The writer would also express his thanks to the Royal Commission for the Exhibition of 1851 for the studentship that has been held during this investigation.

REFERENCES.

Cohnheim (1909). *Z. physiol. Chem.* **59**, 239.
—— (1912). *Z. physiol. Chem.* **76**, 293.
Fiske and Karsner (1913). *J. Biol. Chem.* **16**, 399.
Folin and Berglund (1922). *J. Biol. Chem.* **51**, 395.
Folin and Denis (1912, 1). *J. Biol. Chem.* **11**, 87.
—— —— (1912, 2). *J. Biol. Chem.* **11**, 161.
Griesbach and Oppenheimer (1913). *Biochem. Z.* **55**, 329.
Henriques (1923). *Z. physiol. Chem.* **130**, 39.
Horodynski, Salaskin and Zaleski (1902). *Z. physiol. Chem.* **35**, 246.
Levene and Meyer (1913, 1). *J. Biol. Chem.* **15**, 475.
—— —— (1913, 2). *J. Biol. Chem.* **16**, 555.
Lewis (1912). *J. Biol. Chem.* **13**, 347.
Lewis and Root (1921). *J. Biol. Chem.* **50**, 303.
Luck (1924). *Biochem. J.* **18**, 679.
Nash and Benedict (1921). *J. Biol. Chem.* **48**, 463.
—— —— (1922). *J. Biol. Chem.* **51**, 183.
Nencki, Hahn, Massen, Pawlow (1896, 1). *Arch. exp. Path. Pharm.* **32**, 161.
Nencki, Pawlow, Zaleski (1896, 2). *Arch. exp. Path. Pharm.* **37**, 26.
Neubauer and Fisher (1910). *Z. physiol. Chem.* **67**, 230.
Rohde (1918). *J. Biol. Chem.* **36**, 467.
Salkowski (1880). *Z. physiol. Chem.* **4**, 107.
v. Schroeder (1882). *Arch. exp. Path. Pharm.* **15**, 364.
Van Slyke (1913). *J. Biol. Chem.* **16**, 187.
Van Slyke and Cullen (1914). *J. Biol. Chem.* **19**, 220.
Van Slyke and Meyer (1913). *J. Biol. Chem.* **16**, 197.

CVI. AMMONIA PRODUCTION BY ANIMAL TISSUES *IN VITRO*.

II. THE DEMONSTRATION OF UREASE IN THE ANIMAL BODY.

By JAMES MURRAY LUCK.

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(*Received June 14th, 1924.*)

In the preceding paper [Luck, 1924] it was shown that a specific tissue mixture was capable of liberating a marked amount of ammonia when acting on totally hydrolysed gelatin. It was also shown that the "amide N" as commonly found in the reaction products of the liver preparations was absent from the mixed gastric mucosa-liver preparations. In continuing the investigation of the particular mechanism involved, three problems presented themselves:

(1) The specificity of the liver factor.

(2) The action of one factor on the reaction product of the second factor.

(3) The presence or absence of urea in the reaction products of mixed gastric mucosa-liver preparations.

The technique, general procedure, and methods of analysis were the same as described in the first paper of this series [Luck, 1924].

Exp. 1. The tissues used were those of a male dog of 9 kilos. The results are recorded in Table I.

Table I.

Substrate totally hydrolysed gelatin	Nitrogen per 5 cc. (cc. *N*/10) Ammonia N (nett)	Amide N (nett)
1. Muscle (*A*)	0·07	0·08
2. „ (*A*)	0·10	0·02
3. Liver (*B*)	0·30	—
4. „ (*B*)	0·25	—
5. „ (*B*)	—	—
6. „ (*B*)	—	—
7. Intestinal mucosa (*C*)	0·05	—
8. „ „ (*C*)	0·07	—
9. Gastric mucosa (*M*)	0·10	0·00
10. „ „ (*M*)	0·08	0·05
11. „ „ (*M*)	—	—
12. „ „ (*M*)	—	—
13. Kidney (*Q*)	0·07	—
14. „ (*Q*)	0·05	—
15. *M* + *A*	0·10	—
16. *M* + *A*	0·08	—
17. *M* + *B*	2·28	—
18. *M* + *B*	2·33	—
19. *M* + *B*	2·25	0·13
20. *M* + *B*	2·26	0·05
21. *M* + *C*	0·64	—
22. *M* + *C*	0·58	—
23. *M* + *Q*	0·67	—
24. *M* + *Q*	0·60	—

Substrate totally hydrolysed gelatin	Nitrogen per 5 cc. (cc. *N*/10) Ammonia N (nett)	Urea N (nett)
25. 2 cc. *M* + 1 cc. *B*	1·99	−0·03
26. 2 cc. *M* + 1 cc. *B*	2·18	0·05
27. 2 cc. *M* + 0·5 cc. *B*	1·70	−0·02
28. 2 cc. *M* + 0·5 cc. *B*	1·65	0·03
29. 2 cc. *M* + 0·2 cc. *B*	1·65	0·07
30. 2 cc. *M* + 0·2 cc. *B*	1·65	0·00
31. 2 cc. *M* + 0·1 cc. *B*	1·80	—
32. 2 cc. *M* + 0·1 cc. *B*	1·99	—
33. 1 cc. *M* + 2 cc. *B*	2·30	0·00
34. 1 cc. *M* + 2 cc. *B*	2·18	0·02
35. 0·5 cc. *M* + 2 cc. *B*	2·18	—
36. 0·5 cc. *M* + 2 cc. *B*	2·20	—
37. 0·2 cc. *M* + 2 cc. *B*	2·16	—
38. 0·2 cc. *M* + 2 cc. *B*	2·20	—
39. 0·1 cc. *M* + 2 cc. *B*	2·26	0·01
40. 0·1 cc. *M* + 2 cc. *B*	2·16	0·02
41. *B*	0·30	2·26
42. *B*	0·25	2·20

From these results it may be observed that not only is ammonia liberated by mixtures of the gastric mucosa and liver extracts, but also in smaller but definite amounts by gastric mucosa + intestinal mucosa, and gastric mucosa + kidney extracts. It should also be noted that while urea is found in the reaction products of the liver preparations (tubes 41, 42) it is absent from the reaction products of the gastric mucosa + liver preparations (tubes 25–40). It may also be observed that the urea nitrogen in tubes 41, 42 is equal in quantity to the ammonia nitrogen in tubes 33–40. Either of two conclusions might therefore be drawn from the facts so far recorded:

(1) The gastric mucosa of the dog contains urease.

(2) The gastric mucosa of the dog contains a factor which inhibits the formation of urea (from ammonium salts).

These possibilities have been investigated in Exp. 2.

Exp. 2. A male dog of 11 kilos. was used for the preparation of the tissue extracts. After the addition of the substrates, the volumes were adjusted to 9 cc. and dilute soda added until the contents were neutral to phenol red. 0·5 cc. of toluene was added to each as an antiseptic and the tubes maintained at 37° for 5 days. Ammonia only was estimated—the entire contents of the tubes being washed into the aeration cylinders without previous heat coagulation and filtration. The results are recorded in Table II.

Table II.

	Ammonia nitrogen (cc. $N/10$)
1. 5 cc. urea (0·02 molar) + water	0·00
2. 5 cc. urea + water	−0·02
3. 5 cc. urea + 2 cc. gastric mucosa (*M*)	1·90
4. 5 cc. urea + 2 cc. gastric mucosa (*M*)	1·85
5. 5 cc. urea + 2 cc. gastric mucosa + 2 cc. liver	1·95
6. 5 cc. urea + 2 cc. gastric mucosa + 2 cc. liver	1·98
7. 8 cc. filtered, boiled, reaction product (tube 5, Exp. 1) + 2 cc. *M*	4·23
8. 8 cc. filtered, boiled, reaction product (tube 6, Exp. 1) + 2 cc. *M*	4·00
9. 8 cc. filtered, boiled, reaction product (tube 11, Exp. 1) + 2 cc. liver	1·08
10. 8 cc. filtered, boiled, reaction product (tube 12, Exp. 1) + 2 cc. liver	0·98
11. 5 cc. arginine dinitrate (0·03 molar) + 2 cc. *M* + 2 cc. liver	3·10
12. 5 cc. arginine dinitrate + 2 cc. *M* + 2 cc. liver	2·98

From the results of this and preceding experiments it is concluded that the gastric mucosa of the dog contains urease. From Exp. 1 it may be seen that even 0·1 cc. of a 10 % suspension of the gastric mucosa contains sufficient urease to catalyse the hydrolysis of 5 cc. of 0·02 *M* urea. The ammonia formation that was observed in the gastric mucosa + intestinal mucosa and gastric mucosa + kidney mixtures arises from the hydrolysis of the urea which the intestinal mucosa and kidney are capable of producing. Inasmuch as the intestinal mucosa and kidney contain arginase (though in smaller quantity than the liver), it is suggested that the mechanism investigated and recorded in these papers is as follows.

The arginase contained in the liver catalyses the formation of urea and ornithine from the arginine contained in the substrate. The urea thus formed is broken down to ammonium carbonate by the urease in the gastric mucosa.

As the finding of urease in the dog was quite unexpected, it was a matter of some interest to examine the stomachs of other animals for urease. The procedure adopted was as follows.

As soon as possible after the excision of the stomach, the organ was opened and thoroughly washed with cold running water. The stomachs of the sheep and bullock, owing to the papillary nature of the surfaces, were thoroughly washed and brushed. A portion was then scraped with a knife, the tissue thus removed being used for the urease tests. In the dog and cat, the material was then ground with washed sand and the required quantity of phosphate buffer of p_H 7·45. The material prepared from the sheep and bullock was so finely divided that grinding with sand was not considered necessary.

For the qualitative tests, the material to be examined was suspended in 2 cc. of water in approximately 10 % suspension. 3 cc. of 0·02 *M* urea were added with a few drops of phenol red and very dilute sodium hydroxide till neutral. Subsequent reddening of the solutions through the production of ammonium carbonate was considered to be a positive indication of the presence of urease.

For comparative quantitative estimations, the tissue material was weighed and suspended in 10 parts of phosphate buffer solution of p_H 7·45 at a temperature of 42°. The suspension was then kept at 42–43° for 20 minutes. 5 cc. were then added by pipette to 6 cc. of 0·02 *M* urea in aeration tubes, the whole being at a temperature of 42°. At the end of 30 minutes, caprylic alcohol and potassium carbonate were added and the ammonia estimated by 20 minutes' aeration into standard acid.

The results are recorded in Table III. Duplicate estimations were made throughout.

It may therefore be concluded that urease is contained in the animal body. Of the animals examined, the gastric mucosa of the Carnivora gave the most active preparations. The distribution of the enzyme in the stomachs of the Ruminantia is puzzling but quite definite. It should be noted that no urease could be demonstrated in that portion of the stomach of the Ruminantia in which peptic digestion takes place (the abomasum). No urease could be found in the gastric mucosa of the Rodentia.

Little can be said regarding the possible physiological significance of urease in the animal body. The increased ammonia excretion that has been observed in acidosis in animals (though not in the rabbit) and the corresponding quantitative decrease in the urea excretion could be explained if we assumed that the ammonia came from urea through the carefully regulated action of urease.

The finding of urease in the gastric mucosa explains the results recorded in the preceding paper, but also demonstrates that ammonia formation from α-amino nitrogen was not observed in any of the tissues examined under the conditions of those experiments. The suggestion is that the true deamination of α-amino acids by physiological means requires the survival or growth of the tissue under examination.

Table III.

Tissue examined	Urease tests: Qualitative	Urease tests: Quantitative — cc. $N/10$ ammonia after 30 minutes	Urease tests: Quantitative — % urea decomposed
RAT STOMACH			
Oesophageal area	−	.	.
Pyloric area	−	.	.
RABBIT STOMACH			
Oesophageal area	−	.	.
Pyloric area	−	.	.
GUINEA-PIG STOMACH	−	.	.
SHEEP STOMACH	.	0·10	4
Rumen	+	0·10	4
Psalterium	+	.	.
Reticulum	+ + +	0·83	34
		0·80	33
Abomasum	−	.	.
BULLOCK STOMACH		0·78	32
Rumen—anterior extremity	+ + +	0·80	33
Rumen—posterior extremity	+ + +	0·80	33
		0·75	31
Psalterium	+	.	.
Reticulum	+	0·13	5
		0·16	6
Abomasum	−	.	.
CAT STOMACH	+ + +	1·58	66
		1·90	79
DOG STOMACH	+ + +	2·05	85
		2·15	90
GOAT STOMACH			
Rumen	+ + +	.	.
Reticulum	+ + +	.	.

To Professor Hopkins the writer would express his thanks for much helpful criticism, and to the Royal Commission for the Exhibition of 1851 for the studentship that has been held during these investigations.

REFERENCE.

Luck (1924). *Biochem. J.* **18**, 814.

CVII. STUDIES IN BACTERIAL NUTRITION. II.

By HUGH ROBINSON WHITEHEAD.

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(*Received June 6th, 1924.*)

In a previous paper [1923] the separation of a tryptic digest of caseinogen into two constituents by means of phosphotungstic acid was reported. Two more experiments bearing on the same phase of the problem are now described.

Effect of autoclaving caseinogen stock broth.

Prolonged autoclaving changes some of the substances essential to streptococcal growth and it was of interest to determine whether (i) the reaction of the medium affected its heat stability, and (ii) the substances changed were those in the precipitate or filtrate portion of the broth. Three tubes were prepared, each containing 25 cc. of undiluted stock broth, adjusted to p_H 6·5, 7·5 and 8·5 respectively, and identified as *A*, *B* and *C*. The tubes were heated in steam at 117° for 20 minutes daily for 25 days, samples being taken at 5-day intervals, adjusted to p_H 7·5, diluted 1 in 3 with distilled water, and distributed in smaller tubes for testing growth. A flocculent precipitate, presumably of phosphates, appeared in tube *C* on the 9th day and in the other two tubes on the 14th day. At the end of 25 days a small tube representing each sample was sown with a suspension of the *Streptococcus*. Growth could not be judged accurately because of the flocculent precipitate in some tubes, but where a reasonable growth occurred the characteristic flakes of agglutinated streptococci appeared on the sides of the tube.

There was no apparent growth in the 20-day samples from *A* and *B* or in the 15-day sample from *C*, although all the tubes gave growth on subculturing on to agar. In the 10-day samples from *A* and *B* the growth was equal to that in the control, while the 10-day sample from *C* showed very poor growth. Thus the broth was definitely much less stable (from the point of view of streptococcal growth) in the more alkaline tube *C*.

The original samples *A*, *B* and *C* after being autoclaved for 25 days were filtered, adjusted to p_H 7·5 and tested for growth with the addition of the phosphotungstic precipitate and filtrate portions of caseinogen broth. In this case growth was fairly accurately determined by opacity since no precipitate was present.

Reaction during autoclaving p_H	+ Precipitate	+ Filtrate	Broth alone	Control
6·5	+ + + +	+ +	+ +	+ + + +
7·5	+ + + +	+ +	+	
8·5	+ + +	+	+	

Concentration of broth 1 in 3. Concentration of precipitate or filtrate equivalent to 1 in 3.

The results indicate that the substances destroyed by heat are those contained in the portion precipitable by phosphotungstic acid, and it seems probable that the effect which prolonged autoclaving produces is hydrolysis of some of the more complex compounds in the broth. This, coupled with the fact that the total hydrolysis of caseinogen by means of acid does not produce a medium suitable for the growth of *Streptococcus*, may be taken as evidence that some substance more complex than an amino acid is essential for the growth of the organism.

Effect of adding amino acids to the precipitate portion of the broth.

It was suggested that some amino acid might be the activating principle contained in the filtrate portion and to test this the following amino acids were added to the precipitate portion: glycine, alanine, leucine, tyrosine, tryptophan, histidine, aspartic and glutamic acids. The concentration of the precipitate portion was made equivalent to 1 in 3 stock broth, and the concentration of amino acid was 0·1 % (except tyrosine which was used in saturated solution). On sowing with the *Streptococcus* the result was quite negative in every case as it was also in a solution containing the precipitate portion and all eight acids in 0·01 % concentration. This does not exclude the possibility of some other, possibly unknown acid, being the activating agent, but it is quite evident that the amino group as such is not sufficient.

Extraction by butyl alcohol.

Dakin [1920] has devised and applied a method for the analysis of the products of protein hydrolysis, using butyl alcohol as a solvent, and it was thought that this might prove of some use in elucidating the problem of the substances used by the *Streptococcus* in caseinogen broth. An apparatus similar to that of Dakin was used and various batches of caseinogen stock broth were subjected to continuous extraction by butyl alcohol *in vacuo* at a temperature of 45–50°. By this method the broth is separated into three classes of substances:

(A) Substances quite insoluble in butyl alcohol and therefore remaining in the aqueous solution. According to Dakin the diamino and the dibasic acids are included in this portion.

(B) Substances extracted by butyl alcohol but insoluble in ethyl alcohol. Most of the simple amino acids fall under this head.

(C) Substances soluble in butyl and in ethyl alcohols. Proline and hydroxyproline are the type substances in this class.

The extraction proceeds rapidly at first and a cream-coloured deposit of amino acids separates in the butyl alcohol flask; the deposit is filtered off at intervals to avoid bumping. In the case of caseinogen broth however, which, unlike the solutions used by Dakin, is not completely hydrolysed, the extraction soon slows down and it was found that continuous extraction for 100 hours was necessary before no further appreciable quantity of matter was extracted. Even then it was probable that the extraction was incomplete.

After extraction for 100 hours the whole of the butyl alcohol was separated from the aqueous solution and filtered to remove the deposit, which was then washed with fresh butyl alcohol and with ethyl alcohol. The butyl alcohol containing soluble substances was evaporated *in vacuo* to avoid decomposition and the yellowish deposit remaining was extracted with boiling ethyl alcohol, any insoluble portion being filtered off, washed and added to the solid amino acids *B*. The aqueous portion and the alcoholic solution were both evaporated and finally all three portions were separately redissolved, adjusted to p_H 7·5, and brought to the volume of the original broth. The three solutions were termed *A*, *B* and *C* according to the scheme given above. *A* and *B* were both brownish in colour and *C* was light yellow; only *B* had the sweetish amino acid smell characteristic of caseinogen stock broth. In two cases the total nitrogen and amino nitrogen of all three solutions was determined in order to get some idea of the class of nitrogenous substances present in each solution.

	A		*B*		*C*	
	% N	% Amino N	% N	% Amino N	% N	% Amino N
Broth (Nov. 1923) digested 10 days	0·603	0·369	0·338	0·281	0·105	0·038
„ (Jan. 1924) „ „	0·623	0·317	0·309	0·246	0·077	0·044

Amino nitrogen was determined by Foreman's [1920] method.

The following table gives a summary of the protein reactions by stock broth and by solutions *A*, *B* and *C*.

	Broth	*A*	*B*	*C*
Presence of phosphorus	++++	++++	+	–
Biuret	++++	++++	–	–
Xanthoproteic	No precipitate with HNO_3. Yellow colour → orange on making alkaline			
Millon's	++++	++++	++++	–
Glyoxylic	++++	–	++++	++++
Molisch's	–	–	–	–
Saturated with ammonium sulphate	Precipitate	Precipitate	Slight precipitate	Slight precipitate

All three solutions gave a precipitate with phosphotungstic acid though the bulk was small in *B* and *C*; this shows, however, that some peptide bodies must be extracted by the butyl alcohol since in Dakin's experiments the diamino acids lysine, arginine and histidine were shown to be left unextracted in the aqueous solution. The presence of phosphorus in *B* seems to indicate that some of the phosphates in the broth are still in organic combination.

Growth of the Streptococcus. The three solutions were prepared in small tubes as usual, being diluted so that the concentrations were proportional to stock broth 1 in 3, and sown with equal amounts of the *Streptococcus*. The results in general were remarkably constant.

A	*B*	*C*	*AB*	*AC*	*BC*	*ABC*	Control
+	±	–	+++	+++	++	++++	++++

The incompleteness of the separation was shown by the fact that doubling the concentration of *A* caused a growth of + + +, while doubling *B* caused very little difference in growth. Therefore, the essentials for growth of the

organism are all present in *A* though with some insufficient concentration as is shown by the increase in growth obtained when the other solutions are added.

Trytophan is definitely excluded as an essential growth factor for the *Streptococcus* since solution *A* gives a negative glyoxylic test. Also the results afford further evidence that concentration of amino N is not the deciding factor in the growth of the *Streptococcus* and that specific substances are essential. On the other hand, *B. coli* grew in all three solutions, approximately according to the nitrogen content.

Several attempts were made to separate each of the solutions *A*, *B* and *C* into two fractions by means of precipitation with phosphotungstic acid, but unfortunately without success so far as streptococcal growth was concerned.

The division into six solutions could be carried out so that very little growth-producing power was lost but the separation was not sufficiently clear-cut to enable any useful information to be gained. The separation was particularly incomplete in solutions *B* and *C*, since the phosphotungstic precipitate and filtrate portions of these all seemed capable of giving increased growth when combined with *A*. As mentioned before, the amounts of precipitable substance in *B* and *C* were small, and this may account for the incomplete precipitation. The filtrate portion of solution *A* appeared to be the more important fraction.

Undoubtedly other methods of protein analysis will have to be applied before any clear light is thrown on the subject of bacterial growth substances. The main difficulty from the chemical point of view is the extremely small amount of matter which is sufficient to cause growth of an organism. It has been found, for instance, that the *Streptococcus* will grow in an emulsion of caseinogen unless the protein has been purified by repeated precipitation and that trypsin acting on purified caseinogen for a very short time liberates sufficient substance to cause growth of an organism. Experiments bearing on this point will probably be reported later.

Under these circumstances it is certain that no method, which does not give the accurate separation characteristic of inorganic chemistry, will give absolutely clear-cut results when tested from the growth-producing standpoint.

REFERENCES.

Dakin (1920). *J. Biol. Chem.* **44**, 499.
Foreman (1920). *Biochem. J.* **14**, 451.
Whitehead (1923). *Biochem. J.* **17**, 742.

CVIII. ON THE CONCENTRATION-ACTION CURVE OF INSULIN PREPARATIONS, AND ON ANTI-INSULIN.

By SAMUEL ELZEVIER DE JONGH.

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(*Received June 10th, 1924.*)

In our laboratory we had to determine the strength at which the commercial preparations of insulin made in Holland had to be adjusted before they were brought into commerce. We then met with a peculiar fact, which has become the starting point of the following considerations. Of a new batch 1 cc. of a certain dilution was injected into two rabbits. Both animals showed convulsions and their blood sugar was reduced below 0·045 %, which is to be considered, when testing on rabbits, as the effect of at least three old clinical units of insulin. Two other animals, which were injected with 0·6 cc., did not show any convulsions but their blood sugar was reduced to 0·053 % and 0·046 % respectively. The minimal convulsant dose might then be supposed to lie between these two doses, and indeed 0·8 cc. did not cause any convulsions in two animals, but it did reduce the blood sugar below 0·045 %. So far the testing proceeded quite normally, and in the absence of more exact determinations 0·8 cc. of this dilution was preliminarily considered to be the minimal convulsant dose.

We had come so far, when, for reasons which it is not necessary to mention here, we had to inject a quantity of 1 unit into some rabbits. For this purpose we took a solution of this same preparation which was three times weaker than that referred to above and again used 0·8 cc. *Both animals soon showed convulsions* and a corresponding reduction of blood sugar. This fact, which seemed incredible at first, was repeated with eight rabbits in all, and these all behaved as is usual after injection of a convulsant dose of insulin. Details of the further exact standardisation of this preparation will not interest the reader. Briefly stated, the preparation appeared to possess two convulsant doses, between which there was a less effective zone, and of which the upper one came to 300 % of the lower one. This phenomenon has since then been observed with indubitable certainty in various batches. The schematic curve of the reduction of blood sugar by successive doses of insulin for one such preparation is shown in Fig. 1.

Each of these preparations corresponds more or less exactly to this scheme, because at that time we were unable to use enough rabbits to exclude individual influences entirely.

Therefore I have plotted in a curve the results obtained with 17 preparations of different degrees of purity, which all showed the double convulsant dose, on

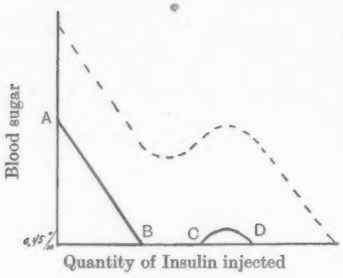

Fig. 1.

the 217 rabbits, which were used for their standardisation (Fig. 4, p. 838). This was done by marking off on the ordinate the lowest blood sugar concentration in parts per thousand, while on the abscissa the quantity of injected insulin is expressed in old clinical units. Here again we considered as three units (= a convulsant dose) the smallest dose with which the convulsion limit might be attained. Now I have taken the average of the lowest blood sugar concentrations of all animals that have been injected with less than $\frac{1}{2}$ unit, and marked it off on the vertical zero line. The average of the animals which we injected with $\frac{1}{2}$ to $1\frac{1}{2}$ units is marked off on the vertical line, corresponding with 1 unit and so on. With each point thus obtained the number of animals is given from which the average blood sugar has been taken. Wherever that number was very small (3) I have connected the adjacent points by a dotted line. There is a striking resemblance between this curve and that of Fig. 1. Only the second elevations (*CD*) of the various batches accumulate more horizontally than vertically. This is quite intelligible as with one preparation the second convulsion limit is only 200, with the other sometimes even 400 % of the lower one. I particularly wish to draw the attention to the rectilinear course of the curve of 0–3 units as I shall discuss its importance later.

For the sake of completeness I must point out that, to save space, the curve has not been continued further to the right than to the upper convulsant dose. Hence the total number of the rabbits whose reactions are recorded in the curve does not amount to 217.

Connected with these phenomena it was observed that the clinicians, who are kind enough to test the preparations on patients before the factory hands them over to the trade, always complained of insufficient activity especially with these batches[1]. Adhering to our definition that the clinical unit is one-third part of the *smallest* dose which is able to cause convulsions and reduction of blood sugar to 0·045 % in a rabbit of about 2 kg. the specimens for the clinics were diluted according to the "lower" convulsion dose. The information from the clinicians was always such, that we definitely had to assume as three units that quantity which we generally call the upper convulsant dose. In

[1] The insulin placed on the market, was then made 2–4 times stronger, according to need, so that these phenomena cannot be quoted as an explanation for eventual disappointment experienced with commercial insulin.

any case the deviation was no more than we also observed in the clinical data before we had observed the two convulsant doses. As these two convulsant doses deviated from 100 to 300 %, the continual correspondence of the upper dose with the effect on patients cannot be said to happen by chance.

So far as to facts. Let us now try to give a plausible explanation of these strange phenomena.

1. *How can the two convulsant doses be accounted for?*

We can say without exaggeration that the straight line has always been taken for granted as the concentration action curve of insulin, for the unit is a biological one and so it does not concern the quantity but the effect. When a preparation is labelled 1 cc. = 20 units, it is already implied that 2 cc. are supposed to have the double effect of 1 cc. As 2 cc. must necessarily contain twice as much insulin as 1 cc., concentration and effect are directly proportional and the curve is a straight line (Fig. 2 *AB*). This supposition seems to accord

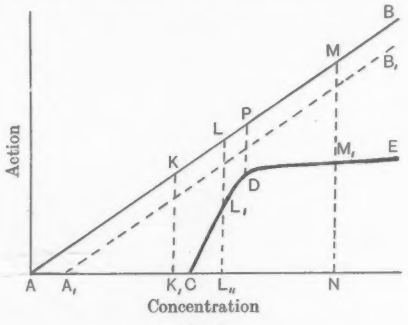

Fig. 2.

with the facts as there have never been any complaints from the clinical side that a greater number of cubic centimetres of the same preparation did not answer to what was expected from the effects of 1 cc.

The above-mentioned facts which we observed on rabbits during the period of August–November, 1923, deviated considerably from this supposition.

This becomes intelligible if we assume an inhibiting substance to be present in the solution which only begins to act with larger doses. The latter is quite possible if this inhibiting substance has a higher limit below which no action is detectable than has insulin, or even when the limit is the same, if the concentration of the inhibiting substance in the solution is less than that of the insulin[1].

Now in order to dominate the insulin, the CA curve[2] of our hypothetical second substance must rise more quickly than that of the insulin. If the height KK_1 indicates the effect of three old clinical units we can easily infer from

[1] For the insulin too has such a limit. Strictly speaking, the curve would consequently be AA_1B_1 and not AB.

[2] Concentration-action curve.

the course of both curves that with L the effect LL_1 (the perpendicular distance between the two curves, which indicates the effect of the mixture) corresponds to less than one convulsant dose, this perpendicular being shorter than KK_1. If, however, the effect of the insulin is to increase again with still greater doses, the curve of the inhibiting substance must bend towards the horizontal (Fig. 2 *D*). If the perpendicular MM_1 is as long as KK_1 the upper convulsant dose is near point N. Now a curve answering to these conditions has been realised in the adsorption curve, a line, which, as a concentration-action curve, is known in pharmacology. So the appearance of two convulsant doses can be accounted for by assuming that the preparations with a double convulsant dose contain two substances which oppose each other. Insulin itself in its CA curve follows the straight line of the partition coefficient; the inhibiting substance follows the adsorption curve (Fig. 3).

Fig. 3.

For the sake of brevity I shall henceforth speak of anti-insulin (AI) without intending to say anything about the character of the inhibiting substance. It certainly is not an anti-toxin.

2. *Why do we not find two convulsant doses in every batch of insulin?*

In other words: must we suppose the anti-substance to be absent when we do not observe two convulsant doses? The answer is quite simple. The two relative concentrations are not always equal in the solution. If it contains relatively more anti-insulin (AI), the lower convulsant dose disappears. For then the entire AI curve (Fig. 2) moves to the left, and as soon as point C comes to the left of K_1 the lower convulsant dose disappears, as in that case the perpendicular distance of the two curves is not as long as MM_1 in any point to the left of N. The presence of AI can then be inferred from the blood sugar curve which follows the dotted line (Fig. 1) repeating the form of our first curve on a higher level.

But also when the anti-insulin proportionately decreases, only one convulsant dose is observed, viz. exclusively the lower one. For then point C is so far to the right that the minimum perpendicular distance of the two curves PD is always greater than the convulsant dose. In observations on rabbits we can then at most get an indication about the presence of the AI, for example, by three convulsant doses only producing one single convulsion within the time limit, perhaps even no convulsions at all but only a reduction of blood sugar to below 0·045 %.

Now we can still ask the question: how is it possible that in the series of preparations following each other quickly, the double convulsant dose has appeared so suddenly and frequently? This will most probably be connected with a change of manufacture which was introduced just then, where the same process of purification was applied three times instead of twice, to decrease the solids. In doing this more anti-insulin than insulin seems to be lost according to comparative curves, and consequently the curve of the AI has moved to the right, thus making two convulsant doses possible, and consequently therefore a large discrepancy between clinical and biological standardisation.

So we must suppose that in previous batches there was relatively even more anti-insulin in the preparations, but that it escaped our observation by the two curves practically covering each other.

At the same time these considerations indicated how it might be possible to avoid the unfavourable action of AI. By purifying once more or even oftener in the same way, the AI always decreases relatively (the AI curve always goes more to the right). One has to bear in mind that the AI curve moreover must proceed relatively less steeply. If point *C* lies very far to the right, the upper convulsant dose will not only disappear, but practically we cannot speak of the presence of AI any longer. In fact, we never saw any indication of the presence of AI in later preparations that had been purified four or five times. But also by applying other methods of preparation known from the literature (short heating, making use of the selective action of substances which flocculate albuminoids, such as picric acid according to Dudley, $(NH_4)_2SO_4$ according to Shaffer, NaCl according to Murlin, and so on) we could avoid the appearance of AI with ever greater certainty. After November, 1923, all preparations we had controlled were free from it.

3. *How can it be explained that insulin always has an effect on man according to the upper convulsant dose?*

In order to make this intelligible one must bear in mind that, if there are two convulsant doses, point *C* cannot be far to the right of point *K*. If that distance is slight, the greatest insulin effect which can be obtained in this zone ($\pm$ 4 units) is undetectable in most human patients.

If we try to give more the AI begins to act. In rabbits, however, these 4 units already produce the maximum effect.

4. *In support of the theory* developed above, we have the fact that under the lower convulsant dose, where theoretically we may not suppose an anti-insulin effect, the CA curve of the insulin really is a straight line. We have observed this several times and in the scheme (Fig. 1) as well as in the curve (Fig. 4) it has been shown by the steeply descending straight line which represents the blood sugars 2 hours after the injection of increasing insulin doses.

In an attempt to eliminate by conversion the deviations which occur on account of the unavoidable differences in the weights of the rabbits, we got still nearer to this straight line. So in this zone the concentration-action curve

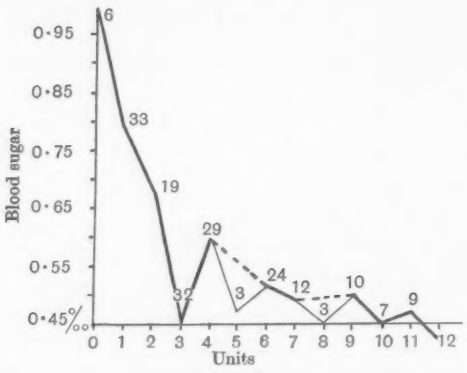

Fig. 4.

of the insulin is certainly a straight line. Now if this holds good for the only part of the CA curve that happens to be controllable we have no reason to suppose that it should not also apply to the further part. We can, of course, hardly speak of the CA curve after the convulsion dose, but when we once more consider the dotted line of Fig. 1 from this point of view, the supposition that its elevation is caused by a second substance becomes very probable indeed. Why then should not the same reasoning apply to the uniform curve with the two convulsion doses?

Finally one might suppose that not a constituent part of the solution but a reaction product of the body, appearing when greater doses are injected, causes these phenomena. But then the connection between the upper convulsant dose and the clinical action, the existence of which cannot be denied, would be incomprehensible.

Summary.

1. The CA curve of insulin, with regard to blood sugar, seems to be a straight line; that of anti-insulin an adsorption curve.

2. In the "Insulin Neerland.," as it was issued some months ago ("purified three times") there was present an inhibiting substance. This substance was called anti-insulin for the time being, which does not imply that it is identical with or akin to other constituents or fractions as having the same effect and the same name, which have often been described, but which mostly either appear at a very early stage of the preparation or whose antagonistic effect can be compared with that of trypsin. This anti-insulin caused (*a*) the phenomenon of the double convulsant doses, (*b*) the resultant enormous differences between experiments on rabbits and on diabetics. As preparations have only been brought into commerce with the upper convulsant limit, which has always been done in Holland, these phenomena (double convulsant limit) cannot have any influence on clinical activity.

CIX. ESTIMATION OF SUGAR IN THE BLOOD.

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(*Received June 23rd, 1924.*)

The object of this paper is briefly (1) to describe a method of estimating the sugar-content of the blood—a method that is accurate and yet so simple as easily to be carried out by medical practitioners; (2) to reply to Stanford and Wheatley's criticism [1924] of my method of estimating the blood-sugar [1923].

First of all let me say that I am indebted for this criticism in that it has led to a profitable re-examination of the subject; their findings, however, have in no way invalidated the accuracy of the method. The points to which Stanford and Wheatley chiefly direct attention are:

(*a*) That in working with duplicate standards (glucose solutions) made at the same time, and using the Folin and Wu method [1919, 1920] or my technique, results were obtained that often varied very considerably one from another.

(*b*) That my contention that, in dealing with the small amounts of glucose used in the estimations, copper is not quantitatively reduced is fallacious and due to the relative concentration of alkaline copper tartrate being considerably lower than that employed by Folin and Wu and by them.

These workers attribute the diversity of results obtained with duplicate standards to the different temperatures at which the blue colour is produced by the addition of phosphomolybdic acid. They state that it is impossible to obtain concordant figures when the reaction is carried out in the "hot," as the temperature of the last of a batch of tubes is bound to be very much lower than that of the earlier ones. This difficulty does not arise if the tubes are put into the boiling water-bath at 20–30 seconds intervals and each one removed therefrom at the end of 6 minutes and the phosphomolybdic acid added immediately. I always do this and do not fail to get accurate results.

Experiment to show the effect of cooling in the air before adding the phosphomolybdic reagent. 5 cc. of the same weak glucose solution were used in each instance; also 2 cc. of the copper solution and 2 cc. of the phosphomolybdic acid.

Tube No.	Interval (minutes) before adding the reagent	Colorimeter readings against a fixed standard	Readings half-an-hour later
1	0	12·6	12·7
2	1	12·7	12·6
3	2	12·7	12·8
4	3	12·8	12·8
5	4	13·0	12·9
6	5	13·9	13·8
7	7	15·1	15·0

The readings, verified by a number of workers in the laboratory, were taken in each case about 5–10 minutes after the formation of the blue colour. In the case of the first four tubes, full development of colour had taken place within this period, but with the remaining tubes a longer time was required, e.g. about half an hour for the 7 minute tube. Therefore, in using the quantities and the method I employ, no error is introduced by carrying out the reaction in the "hot."

As regards their second point, I would remind them that the relative concentrations of alkaline copper tartrate and sugar in my standard [1923] are the same as those of Folin and Wu, but the absolute concentrations are of course much weaker. I have, however, increased the strength of the copper solution ($\times$ 1·75) so that its concentration, when mixed with the 5 cc. sugar solutions, is equal to that in Folin's method and have repeated the experiments described in the construction of my "curve of correction" [1923]. A copper solution stronger than this has also been employed—one twice the strength instead of 1·75 times. All the estimations have been carried out both in the "hot" and at the 20° recommended by Stanford and Wheatley.

The double-concentration copper solution may be dismissed with the remark that it gave the same results as the "1·75" copper solution (hereafter referred to as "the copper solution").

It was found that the reduced copper solution (7 cc. in vol.) was lowered in temperature to 20° by immersion in cold water for $3\frac{1}{2}$ minutes or less, depending on the temperature of the water; running water will considerably shorten this period. However, I have cooled the solutions in the way Stanford and Wheatley suggest. Some interesting results have emerged from these experiments which will now be described.

In the way indicated in my paper [1923] a duplicate series of 5 cc. solutions, containing amounts of the purest dry glucose ranging from 0·025–0·800 mg., were prepared. 2 cc. of the copper solution were then added and two tubes of equal sugar concentration were simultaneously placed in a vigorously boiling water-bath. At the end of 6 minutes 2 cc. of phosphomolybdic acid were added to one immediately, while the other was cooled to 20° by immersion in cold water for $3\frac{1}{2}$ minutes before the addition of the acid. Both solutions were then made up to the 12·5 cc. mark. On holding them up to the light, the contrast in colour is very striking. The one formed in the "hot" is a pure blue (or a blue with a slight indigo tint), while the "cooled" solution is a blue with a marked greenish tinge in it. It is impossible to match the colours in the colorimeter. Now the first set of colours (the "immediate" colours) can be at once matched easily and accurately in intensity, and also in tint, with my fixed standards in glass. The readings remain the same for at least a couple of hours and then slowly have to be increased to maintain the match. On the other hand, the colours produced by the cooling method change slowly in the deep solutions and comparatively rapidly in those of light intensity, until the colour produced in the hot solution is reached. Thus in the case of two 5 cc.

solutions each containing 0·10 mg. of glucose, the "cooled" one will change from its greenish-blue colour to that of the practically stable "immediate" blue of the "hot" solution in about 20 minutes, while the colour of a "cooled" solution containing 0·80 mg. will require about 2–2½ hours to develop the "immediate" colour. The interesting feature is that the solutions are then equal in colour-intensity. This shows that the degree of oxidation that occurs during a short period of cooling in suitably constructed boiling-tubes may be considered as negligible. The cooling process introduces a time-consuming factor (the slow development of colour) and is apt to lead to error. It is better to treat the solutions in the "hot," avoiding any possible inaccuracy due to oxidation by spacing the times of heating as already indicated. It is worth noting that the colour of the water-cooled solutions may be changed rapidly to that of the "hot" solutions by re-heating in the water-bath. Further, the greenish tint does not seem to occur with similar short periods of air-cooling, presumably because the lowering of temperature is much slower and therefore much less pronounced.

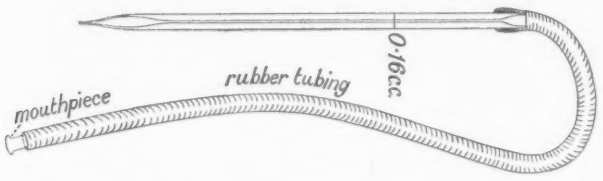
Curve of correction for "found" values. The results of the experiments referred to above show that, using a colorimeter of the Kober or Duboscq type and the new copper solution, quantitative findings are not obtained except for solutions only a little removed in concentration from that of the standard. [See *Table of Correction*, p. 844.] The estimations have been repeated many times and the utmost care exercised on every occasion.

The effect of multiplying the strength of the original copper solution by 1·75 has been to increase the intensity of colour developed for any given amount of glucose. This has enabled me to use a standard containing a smaller quantity of glucose, namely, 0·15 mg. in 5 cc. of distilled water, and has also permitted of the employment of as small an amount of blood as 0·16 cc. The curve is also less divergent from the line of quantitative reduction.

A SIMPLE AND ACCURATE METHOD OF ESTIMATING THE SUGAR-CONTENT OF BLOOD: SUITABLE FOR MEDICAL PRACTITIONERS.

To avoid the expense of the torsion balance and platinum capsule of my previous method, a pipette has been introduced for the collection of the blood. The pipette is similar to that of MacLean; it is smaller and calibrated to measure 0·16 cc. The nose of the pipette is longer and narrower in lumen, while the bore of the stem is also much finer. These modifications enable one readily to collect the blood by means of suction and for this purpose a piece of rubber tubing is attached to the pipette. It is important that the bulb of the pipette be spindle-shaped in order to prevent the trapping of air. The pipettes must be kept thoroughly clean and dry; this can be accomplished by washing successively with water, absolute alcohol and ether, and then passing dry air through them. Clotted blood may be removed by the use of hot nitric acid.

The 0·16 cc. of blood is easily obtained from a single prick of the finger or thumb, preferably the latter, as the blood is less liable to overrun its broad dorsal surface. Swinging the arm and the application of a piece of rubber tubing around the base of the digit ensure an ample supply of blood. *Without delay* the specimen is washed out of the pipette into 7·3 cc. of distilled water which is contained in a short wide test-tube (say 1″ × 3½″).



Before proceeding further, it is necessary to describe the other apparatus required[1]:

(1) Several special resistance-glass boiling-tubes with 7 cc. bulbs and graduated for 12·5 cc.

(2) Pipettes of the following capacities: two 1 cc. pipettes (in $\frac{1}{10}$ths) for the tungstate and H_2SO_4 solutions; two 2 cc. ordinary bulb-pipettes for the copper and phosphomolybdic acid solutions; one 5 cc. pipette for the blood filtrate.

(3) 25 cc. burette.

(4) Water-bath (fairly large).

(5) Whatman starch-free filter papers, No. 30 (7 *cm. diameter*).

(6) The following solutions:

(*a*) 10 % sodium tungstate.

(*b*) $2N/3$ H_2SO_4.

(*c*) *Copper Solution.* Dissolve 70 g. pure anhydrous sodium carbonate in about 500 cc. distilled water in a litre flask. Then add 13·125 g. tartaric acid, and when the latter has dissolved and effervescence has ceased, add 7·875 g. pure crystallised copper sulphate; dissolve without the aid of heat and make up to one litre with distilled water. Keep in a dark-coloured bottle. If any sediment occurs during the first couple of weeks, then transfer the clear solution to another bottle.

(*d*) *Phosphomolybdic acid solution.* 35 g. *pure* molybdic acid are dissolved in 200 cc. of 10 % NaOH and 200 cc. of water added. The whole is now boiled for 20–40 minutes, or longer, until all traces of ammonia have been driven off. Cool and dilute to about 350 cc. with water and then add 125 cc. of phosphoric acid (85 % strength) and water to 500 cc.; 2 cc. of this solution when added to 2 cc. of the copper solution should produce complete decolorisation.

[1] The entire outfit may be obtained from Baird and Tatlock (London), Cross St, Hatton Garden, E.C.

(*e*) *Stock sugar solution.* Dissolve 4 g. of absolutely pure powdered glucose in 1 litre of distilled water, a few drops of toluene being added as a preservative.

The *Standard sugar solution* is prepared by placing 7·5 cc. of the stock solution in a litre volumetric flask and making up to the mark with distilled water. 5 cc. of this solution contain 0·15 mg. glucose.

Solutions (*a*), (*b*), (*c*), (*d*) seem to keep indefinitely while the stock sugar solution will, with ordinary care, remain unaltered for several months.

(7) A reliable colorimeter. For use in general medical practice this need not be an expensive one. Indeed, with a little practice one can tell with the *naked eye*, from the depth of blue colour developed (to be described presently), whether the blood-sugar content is normal or not; for comparison the colour derived from a normal fasting blood or from a sugar solution of known strength (e.g. 0·1 mg. in 5 cc. water) may be employed.

Technique of estimating the blood-sugar. This will be dealt with briefly as details are to be found in my paper referred to.

(*a*) To the solution of 0·16 cc. blood in the 7·3 cc. distilled water are added 0·3 cc. 10 % sodium tungstate and then 0·3 cc. $2N/3$ H_2SO_4. This makes the volumes up to 8 cc. (0·1 cc. being allowed for blood, less evaporation during experiment). A rotatory movement of the test-tube mixes the solutions which turn to a chocolate colour in a couple of minutes. The proteins have now been completely coagulated. The solution is next filtered through the 7 cm. Whatman filter-paper; this must be done carefully in order to avoid the escape of protein, which event would spoil the estimation.

(*b*) 5 cc. filtrate are pipetted into a boiling tube, 2 cc. of copper solution added, and the tube placed for exactly 6 minutes in a vigorously boiling water-bath. The addition of 2 cc. of phosphomolybdic acid solution immediately on withdrawing the tube from the bath produces the blue colour. The standard blue colour for comparison is obtained by treating similarly 5 cc. of the *standard sugar solution.* After standing for about 4 minutes the solutions are cooled under the tap and made up to the 12·5 cc. mark with distilled water. Inversion of the tubes mixes the solutions, which are then compared in a colorimeter. Reference to my paper [1923] will show how the trouble of making standard solutions may be avoided by employing glass discs which match the standard blue. The "7·5 *B*" discs equal in colour intensity the standard blue solution set at 13·7 mm. reading, and as the level is optional, within certain limits, at which one may set the standard the use of the discs means a considerable saving in time and labour. The 13·7 figure has been obtained with the purest glucose and this invests the discs with a further advantage when glucose solutions are of doubtful quality.

An example of the calculation of results: 0·16 cc. sample of blood taken from a normal person (fasting). The colorimeter reading of the unknown blue solution when matched against the standard blue solution, set at 13·7 (or, what is equivalent, against the discs) was found to be 20·3.

The standard colour or the discs (i.e. 13·7) represents 0·15 mg. glucose, therefore the unknown (or 5 cc. of blood filtrate)

$$= \frac{0{\cdot}15 \times 13{\cdot}7}{20{\cdot}3} = \frac{2{\cdot}055 \text{ (a constant factor)}}{20{\cdot}3} = 0{\cdot}101 \text{ mg. glucose.}$$

Now 5 cc. of blood filtrate

$= \frac{5}{8}$ amount of blood taken (0·16 cc.) = 100 cmm.,

$\therefore$ 100 cmm. blood contain

= 0·101 mg. glucose (uncorrected),

$\therefore$ normal "fasting" blood-sugar

= 0·102 % (corrected from Table).

Table of Correction.

Values mg. glucose		Values mg. glucose		Values mg. glucose	
Uncorrected	Corrected	Uncorrected	Corrected	Uncorrected	Corrected
0·020	0·029	0·130	0·130	0·340	0·324
0·030	0·040	0·150	0·150	0·360	0·342
0·035	0·045	0·170	0·170	0·380	0·358
0·040	0·048	0·190	0·190	0·400	0·377
0·045	0·052	0·195	0·195	0·420	0·395
0·050	0·056	0·200	0·199	0·440	0·409
0·055	0·061	0·205	0·204	0·460	0·427
0·060	0·065	0·210	0·209	0·480	0·443
0·065	0·070	0·215	0·213	0·500	0·459
0·070	0·075	0·220	0·218	0·520	0·476
0·075	0·080	0·225	0·223	0·540	0·492
0·080	0·085	0·230	0·228	0·560	0·510
0·085	0·089	0·240	0·237	0·580	0·525
0·090	0·093	0·250	0·246	0·600	0·542
0·095	0·098	0·260	0·255	0·650	0·583
0·100	0·102	0·270	0·265	0·700	0·622
0·105	0·107	0·280	0·274	0·750	0·661
0·110	0·111	0·290	0·283	0·800	0·699
0·115	0·115	0·300	0·291	0·850	0·747
0·120	0·120	0·320	0·307	0·900	0·775

These figures may be plotted as a curve which will facilitate the process of correction.

SUMMARY.

The paper consists in

(*a*) the description of a method of estimating the sugar-content of blood, so simple and accurate as to appear ideal for general medical practice;

(*b*) a refutation of the criticism by Stanford and Wheatley of my article [1923].

REFERENCES.

Stanford and Wheatley (1924). *Biochem. J.* **18**, 22.
Calvert (1923). *Biochem. J.* **17**, 117.
Folin and Wu (1919) *J. Biol. Chem.* **38**, 106.
—— —— (1920) *J. Biol. Chem.* **41**, 367

CX. THE HAEMOLYTIC ACTION OF THE SOAPS.

By ERIC PONDER.

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(*Received June 26th, 1924.*)

In nearly every text-book of physiology the soaps are referred to as haemolytic substances, but the literature contains no quantitative information regarding their action. To supply this deficiency the following investigation was carried out.

Methods and material employed.

(i) *Soaps.* The results recorded in this paper were obtained from the examination of two specimens of sodium stearate, one of potassium stearate, two of sodium palmitate, two of potassium palmitate, three of sodium oleate, and one of potassium oleate. The specimens employed were selected for their purity. The use of impure samples of soap may give rise to very fallacious results, common impurities being alkali and even saponin, the presence of either of which will completely vitiate any results obtained. The soaps were dissolved, in suitable concentration, in 0·85 % NaCl. Experiments were carried out as soon as possible after the preparation of the solutions.

(ii) *Cell suspension.* The cells used were human erythrocytes, collected into citrate, and thrice washed with 0·85 % NaCl. The cells contained in 1 cc. of blood were suspended in 20 cc. of 0·85 % NaCl., the strength of the suspension thus formed being the same as that usually employed in the writer's researches.

(iii) *Methods.* The time-dilution and time-temperature curves for the various specimens of soaps were determined in the manner described in a previous paper [1921]. The curves for percentage haemolysis were obtained by the use of the apparatus already described [1923, 2]. The action of serum on the lysis produced by the soaps was investigated by the methods previously used in connection with the inhibitory effect of serum on saponin and taurocholate haemolysis [1923, 1]. The methods employed for the determination of alterations in size of the cells under the action of the soaps have also been previously described [1922; Ponder and Millar, 1924]. Estimations of the surface tension of the solutions used as lysins were made by the capillary rise method in the usual way.

Results.

The most convenient way in which to present the results is to consider each soap separately. In cases where more than one specimen was investi-

gated, it is to be understood that no discrepancy was found in the results yielded by the various specimens.

It may be as well to point out from the commencement that the soaps are very unsatisfactory as lysins when any data of a quantitative nature regarding their action are required. Being semi-colloids, they exhibit all the phenomena characteristic of their class. Their physical properties show a great tendency to alteration from time to time, the time-factor having to be taken into account in all determinations of their surface tension, their conductivity, and even of their haemolytic activity. As the circumstances which determine their physical conditions are not fully known, it is almost impossible to make two solutions of the same soap which are identical as regards physical state. This difficulty is sufficiently well marked when the soaps are in aqueous solution—when solutions in saline are dealt with, the difficulties are much greater, as the soaps tend to salt out, this change increasing with passage of time. This unsatisfactory state of affairs is perhaps responsible for the absence of any quantitative researches from the literature. The experiments recorded in this communication, are, of course, subject to the same difficulties, and must be judged accordingly. It is, however, possible to obtain a very good idea of the action of these substances, and to make certain quantitative determinations, if care is taken to use freshly prepared solutions, and to take into account the time which elapses between the preparation of the solutions and the performance of the experiments.

Sodium stearate. This soap forms a clear solution when hot, and gels on cooling. In a concentration of 1 in 500, an opalescence appears at 58° in saline solution—at 50° the solution is quite opaque. Gel formation becomes marked at about 40°. This results in great difficulty in making dilutions for haemolytic experiments, and in getting the red cells to mix with the lysin, for, in the cold, even solutions as dilute as 1 in 5000 show gelation.

The following table shows the relation between the dilution of sodium stearate and the time taken for the production of complete haemolysis of 0·4 cc. of cell suspension at 25°. The quantity of lysin used is 2 cc., the dilution being calculated after the addition of the cells.

Dilution	Time in minutes
1000	1
2000	2
4000	4·5
6000	20

From these figures it may be concluded that the haemolytic activity of sodium stearate is of the same order as that of sodium taurocholate.

At low temperatures, haemolysis is very slow—the fact that gelation occurs renders it impossible to form any accurate estimation of the haemolytic activity. At temperatures above 15° it becomes quite possible to estimate the rate of haemolysis, which steadily increases with rise of temperature. The relation between the temperature and the time taken for the production of complete lysis by any concentration of stearate appears to be the hyperbolic

one met with when saponin or the bile salts are used [1921]. Owing to the tendency to progressive opalescence and change of physical state alluded to, it is not easy to obtain either time-dilution curves or time-temperature curves for this soap. Irregularities, which can only be explained by changes in the activity of the haemolysin attributable to its tendency to physical change, are frequently met with, and interfere with the results of these haemolytic experiments.

Owing to the opalescence referred to, which occurs even in dilute solutions, it is impossible to obtain a percentage haemolysis curve by means of the radiometer.

The action of serum appears to be to inhibit the haemolysis produced by sodium stearate, if the serum be added to the soap before the addition of the cells. This action, however, is by no means well marked. If the serum be added to the soap after the latter has been in contact with the cells, no acceleration of haemolysis is to be observed as a rule—in a few cases there was a suspicion that there was an acceleration, but the result is so inconstant and ill-marked that no importance can be attached to it.

Care was taken to look for "periodicity phenomena"—that is, haemolysis in high dilutions when there is no haemolysis produced in more concentrated solutions of the lysin,—such as are observed with sodium taurocholate and human cells, and, according to Pickering and Taylor [1923], with saponin and the cells of the sheep, guinea-pig, and white rat. No such phenomena are observable with sodium stearate.

The alterations in form which the cells undergo are in general similar to those produced by saponin and sodium oleate, but somewhat less marked. Owing to the tendency of the soap to gel, it is very difficult to obtain information on the point, as there is great difficulty in making the necessary photomicrographs: solutions which do not gel at ordinary temperatures are too slow in their haemolytic action to give good results.

It may be mentioned that solutions of sodium stearate in saline tend to become less haemolytic on standing. This is probably associated with the progressive alteration in physical state which they undergo. As a result, freshly prepared solutions must be employed, and even in these, irregularities are met with.

Potassium stearate. This soap possesses properties very similar to those of the sodium soap. It gels in the same way in the cold, and forms clear solutions above 60°. Its haemolytic activity is of the same order as that of the sodium soap, from which it differs in no important way. It is impossible to obtain a percentage haemolysis curve for potassium stearate, owing to the opalescence which appears in solutions of the necessary concentration. The action of this soap is inhibited by serum. No periodicity phenomena are observable, and there appears to be little or no acceleration produced by adding serum to the lysin after it has been in contact with the cell suspension. The loss of haemolytic power on standing is well marked.

Sodium palmitate. This soap shows less tendency to opalesce and to gel than does the stearate, but these properties are sufficiently well marked to make haemolytic experiments very difficult. Solutions are clear above 60°, and show opalescence about 55°. The time-dilution relation at 25° is as follows:

Dilution	Time (min.)
1000	0·5
2000	1·0
4000	2·5
6000	9·0
8000	35·0

The palmitate of sodium is therefore a little more haemolytic than is the stearate. At low temperatures the usual retardation of haemolysis is observed, the rate increasing as the temperature increases. It is not possible to obtain a curve for percentage haemolysis, owing to the opalescence in the solutions used. Serum retards the haemolytic action of the soap, but not to a very great extent—the inhibition produced, moreover, is somewhat variable, so that it is not possible to make a quantitative study of the inhibitory phenomena. This variation appears to be connected with the general instability of physical state which the soap shows. On standing, solutions lose their lytic power considerably.

The alterations in form of the cells are similar to those met with under the action of other soaps: the same difficulty as is encountered in the case of sodium stearate renders the making of photographic measurements very difficult, and rather unreliable.

Potassium palmitate. The results of experiments on this soap may be summarised by stating that its action is almost identical with that of sodium palmitate. Potassium palmitate is no easier to work with than is the sodium salt, as its tendency to opalescence and gelation is as great, if not greater.

Sodium oleate. This soap is the only one of those investigated with which it is possible to obtain satisfactory results, and even in this case considerable irregularity is met with. Solutions in saline of concentrations between 1 in 500 and 1 in 10,000 exhibit a well-marked opalescence, especially after standing for a little while—no gelation, however, occurs in the dilutions required for haemolytic experiments. This opalescence is accompanied by a loss in the haemolytic power, which loss becomes very noticeable after the lapse of some time. Care must therefore be taken to compare samples which have stood for the same length of time, and to make that time as short as practicable.

At 25° the time-dilution relation is

Dilution	Time (min.)
5,000	0·5
10,000	1·5
20,000	3·5
30,000	8·0
40,000	20·0

These figures show that the haemolytic activity of this soap is nearly as great as that of saponin, and considerably greater than that of the other soaps examined, with the exception of potassium oleate.

The time taken for complete haemolysis increases as the temperature is reduced, lysis being very slow at temperatures below 5°. As the temperature rises, the rate of haemolysis increases in a regular manner, the relation between the temperature and the time taken for complete lysis being of the usual hyperbolic type. Some irregularities may be met with at low temperatures if solutions of soap which have undergone alterations of physical condition as a result of standing are employed: if fresh solutions are used, the time-temperature curves are very regular.

The appearance of opalescence in solutions of sodium oleate renders it a difficult matter to obtain a percentage haemolysis curve. Curves which are approximately correct can be obtained, however, with suitable concentrations of soap and cell suspension, and at suitable temperatures, which is more than can be done with any other soap. With a dilution of 1 in 20,000 at 14°, acting on 20 cc. of a cell suspension one-quarter the strength of that described above, the radiometer gave the following relations:

Time (min.)	% haemolysis	Time (min.)	% haemolysis
0	0	5	57
1	1·5	6	76
2	12	7	87
3	21	8	94
4	35	9	100

The corresponding curve is of the usual sigmoid type, and exhibits a slight skewness, the tendency being for a slight acceleration of the reaction towards its completion. The skewness is, in this case, about 0·12, a small amount for curves of percentage haemolysis. In accordance with the general principles considered in a previous paper, we may deduce that serum and haemoglobin have, if added to sodium oleate after this soap has been in contact with red cells, an accelerating effect on its haemolytic action, but that this effect is not well marked. If more concentrated solutions of soap than that mentioned in the above experiment are employed, the skewness in the percentage haemolysis curve becomes almost negligible, so that the curve appears to be a symmetrical one. This also, is what is to be expected from general considerations.

The haemolytic action of sodium oleate is markedly inhibited by small amounts of serum or plasma, if these be added to the soap before the latter comes in contact with the erythrocytes to be haemolysed. The inhibitory action of serum on the soap is not so well marked as it is on saponin, nor is the amount of inhibition produced by a given amount of serum so easily estimated, as considerable irregularities occur. Haemoglobin also has a very slight inhibitory effect. If the serum be added to a mixture of soap and red cells, the inhibition is replaced by an acceleration, as in the case of sodium tauro cholate and sodium glycocholate [Ponder, 1923, 2]. This acceleration is not so well marked, however, as in the case of the bile salts. If the serum be added to the mixture of soap and cells immediately after this mixture has been made, the acceleration is very slight; if, on the other hand, the soap be allowed to complete the lysis of about 50 % of the cells in the absence of serum, the

addition of a small quantity (0·1 cc.) of serum or of haemoglobin solution causes an almost immediate haemolysis of the remainder. The effect of haemoglobin is slight compared with that of serum, or with the acceleration produced by haemoglobin on taurocholate haemolysis. These facts explain the positive skewness of the percentage haemolysis curve for sodium oleate, and also show why this skewness should be relatively slight: it is to be assumed that the haemoglobin released from the cells first haemolysed acts as an accelerating agent, and hastens the lysis of the remaining cells to a slight, but appreciable, extent.

No periodicity phenomena are to be observed with sodium oleate.

The alterations in form which erythrocytes undergo during haemolysis by sodium oleate may be observed readily enough, and are fully dealt with in another communication [1924]. These changes are similar to those met with when saponin is used as a haemolysin—the cells, immediately before lysis, become spheres without an alteration of volume; thereafter the diameter increases slightly, and the cell haemolyses.

Potassium oleate. This soap acts in a manner almost identical with that of sodium oleate, except that it is more difficult to work with, on account of the opalescence developed.

Surface Tension Experiments.

Since it is often suggested that the soaps are haemolytic because they lower surface tension, some experiments on this point were carried out, and may be briefly mentioned here.

Sodium oleate. Estimations of the surface tension of solutions of sodium oleate in saline solution, and in solution in water, confirm the observations (i) that it is very difficult to obtain concordant results, and (ii) that there is no finite value for $d\sigma/dc$ [Milner, 1907].

Sodium palmitate. This soap is stated not to lower the surface tension of water [Bottazzi, 1912]. Estimations made by us do not confirm this observation, for we find that a 1 in 20,000 solution has a surface tension of 53 dynes at 20°, and that slight lowering of surface tension is shown by solutions between the concentrations of 1 in 5000 and 1 in 50,000. Owing to gelation, figures for concentrations greater than 1 in 5000 cannot be obtained.

Sodium stearate. This soap also lowers surface tension. This is shown in the table below, where the soap was in saline solution, and the determinations made at 20°.

Concentration (c)	σ, dynes
1 in 5,000	51·3
1 in 10,000	52·1
1 in 20,000	51·8
1 in 40,000	57·4
1 in 80,000	60·4
1 in 160,000	63·4
Saline	63·4

This table shows that, as in the case of sodium oleate, there is no finite value for $d\sigma/dc$. The surface tension is almost the same for all concentrations above

about 1 in 30,000, and thereafter becomes greater, reaching that of water at a concentration of about 1 in 100,000. The fact that the surface tension of the soaps bears no definite relation to the concentration is an argument of considerable weight against the lytic action being due to a lowering of surface tension, as is frequently stated.

Summary.

Summarising the general results of this investigation, the following conclusions may be drawn. (i) The stearates, palmitates and oleates are haemolytic, the stearates being the least so, and the oleates the most. (ii) There is little difference between the activity of the sodium and the potassium soaps. (iii) The time-dilution curves for these substances are of the usual logarithmic type. (iv) The time-temperature curves are of the usual hyperbolic type. (v) The percentage haemolysis curves cannot be investigated satisfactorily, except in the case of sodium oleate, owing to the opalescence which exists in haemolytic concentrations of the soaps. In the case of sodium oleate, this curve is of the usual sigmoid type, and shows, when suitable concentrations are used, a slight positive skewness. (vi) Serum or plasma, and also solution of haemoglobin, show an inhibitory effect on the lytic action of the soaps, especially on the action of the oleates. (vii) This inhibitory effect may be replaced by an acceleration, if the serum, plasma, or haemoglobin be added to the soap after this has been allowed to act on the cells. Such acceleration is not a very marked one. These facts explain the form of the percentage haemolysis curve for the oleates. The acceleration effect is very ill marked, or even absent, in the case of the stearates and the palmitates. (viii) No periodicity phenomena are observable with the soaps. (ix) The changes of shape which erythrocytes undergo when acted on by these soaps are very similar to the changes produced by saponin and by the bile salts. (x) Solutions of the soaps tend to lose their haemolytic power on standing. (xi) All indications point to the action of the soaps being of the same type as that of sodium taurocholate, and it is probable that they have their effect by attacking the protein components of the erythrocyte.

I have to thank Dr J. A. Hewitt for certain of the specimens of soap used in this investigation.

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REFERENCES

Bottazzi (1912) *Atti R. Accad. Lincei*, 365.
Milner (1907). *Phil. Mag.* **13**, 96.
Pickering and Taylor (1923). *Biochem. J.* **17**, 916.
Ponder (1921). *Proc. Roy. Soc. London*, B, **92**, 285.
—— (1922). *Proc. Roy. Soc. London*, B, **94**, 102.
—— (1923, 1). *Proc. Roy. Soc. London*, B, **95**, 42.
—— (1923, 2). *Proc. Roy. Soc. London*, B, **95**, 382.
—— (1924). *Quart. J. Exp. Physiol.* **14**.
Ponder and Millar (1924). *Quart. J. Exp. Physiol.* **14**, 67.

CXI. A METHOD FOR THE ESTIMATION OF IRON IN SMALL QUANTITIES IN BIOLOGICAL SUBSTANCES.

By MARGARET MARY MURRAY.

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(*Received July 15th, 1924.*)

The general principles of the method are not dissimilar to those in general use, but certain defects in the $KMnO_4$ method can be overcome by the use of a "reductor" instead of the more usual Bunsen valve, previous to titration with permanganate, and titration in an atmosphere of hydrogen makes the method reliable and accurate. The method has been worked out, using blood.

Incineration. 3 cc. of fully oxygenated whole blood are run into a weighed "vitreosil" crucible and accurately weighed. The crucible is then placed without lid in a steam oven for half an hour, or, if time will not permit of this, the blood is dried carefully with a small flame. When dry, the crucible is heated more strongly in order to ash the contents. Small crystals of ammonium nitrate are added from time to time to help combustion.

Strong heating is to be avoided, otherwise iron will be lost, as pointed out by Peters [1912], because if strongly heated the iron residue becomes insoluble. The burning mass should not be allowed to froth up round the sides of the crucible but be kept as compact as possible.

Solution of the iron residue. When combustion is complete the crucible is allowed to cool; about ten drops of pure concentrated hydrochloric acid added and the whole gently heated to dissolve the iron residue, further heating is applied and the contents of the crucible evaporated to dryness.

In order to remove chlorides, since these vitiate the result as pointed out by Barcroft [1914], approximately 1 cc. of pure concentrated sulphuric acid is added to the chloride residue, which is again heated till white fumes are no longer evolved. The contents of the crucible are finally taken up in four successive portions of 3 to 4 cc. of 10 % sulphuric acid.

Reduction of the iron and titration with permanganate. It is in this stage of the proceedings that improvements on former permanganate methods have been introduced. For conversion of the iron to the ferrous state a modified Jones' reductor is used.

The reductor. A white glass filter flask is fitted with a two-holed rubber cork to take the reductor and a burette. The reductor itself is a tube 75 cm.

by 2·5 cm. packed to a length of 30 cm. with "cadmiumised" zinc, that is, zinc which has been soaked in 2 % cadmium sulphate solution for five minutes and then well washed.

Pure zinc is too slow in its action to reduce the iron properly. No trace of zinc must be allowed to pass into the flask; to prevent this happening the narrow part of the tube just above the tap is packed with "melting point" tubing and capped with a plug of glass wool. This type of filter is used because if a close filter is used the process is too slow.

The end of the reductor below the tap is cranked before passing through the cork. The burette is filled with standard permanganate and inserted before reduction begins.

Reduction. The flask is connected to a pump and the solution containing the iron in 10 % sulphuric acid is poured in a steady stream down the reductor, the tap opened and the solution allowed to escape at a slightly slower rate into the flask. The reductor is washed with about 20 cc. of 5 % sulphuric acid. During these proceedings the tap must not be allowed to become unsealed.

Titration. With the pump still going the standard permanganate is run in from the burette and the flask well shaken till the solution takes on a pink tinge. The colour is permanent even though it is faint; the end point is therefore very distinct.

A blank titration is made by running through the reductor 12 cc. of 10 % sulphuric acid and 20 cc. of 5 % acid and titrating. This solution never required more than 0·05 cc. of permanganate, and this amount was deducted from the iron titrations.

Since very small quantities of permanganate make a great difference to the quantity of iron estimated, a 10 cc. burette graduated in $\frac{1}{50}$ths was used. The permanganate employed was tried at various strengths, $N/60$, $N/80$, $N/100$; the end point was quite definite even with the $N/100$ solution. All these solutions were made up immediately before use from a stronger solution which was always standardised. The $N/80$ strength permanganate seemed to be the most convenient.

Peters [1912], in his work on the constancy of the proportion of iron to oxygen in oxyhaemoglobin, used the titanium method and Barcroft [1914] considered that the application of this method made the estimation simpler and more accurate than was possible with the permanganate method. It is doubtful to me whether the method is simpler; it is certainly not a student's method.

The use of permanganate for the estimation of iron in blood was deprecated by Barcroft and Peters because it is vitiated by the presence of chlorides, whereas the titanium method is not: if chlorides, however, are first removed, this criticism does not apply.

Many investigators decry methods which involve titrations with dilute permanganate; no doubt the greatest source of error in these is the indefiniteness of the end point, but titrations done under the conditions described give a perfectly clear-cut end point.

Results.

Series 1.

Cat's blood	Weight of blood, g.	cc. $N/80$	Fe % in blood	Fe % computed from O_2 capacity
A	3·1412	2·00	0·0443	—
B	3·1481	2·05	0·0454	—
C	3·1403	2·00	0·0445	—
		Mean	0·0447	0·0446

Series 2.

Cat's blood	Weight of blood, g.	cc. $N/100$	Fe % in blood	Fe % computed from O_2 capacity
A	3·1295	2·85	0·0510	—
B	3·1819	2·88	0·0506	—
C	3·0536	2·78	0·0510	—
D	3·0752	2·82	0·0513	—
		Mean	0·0510	0·050

Series 3.

Cat's blood	Weight of blood, g.	cc. $N/60$	Fe % in blood	Fe % computed from O_2 capacity
A	3·0124	1·46	0·0452	—
B	3·0812	1·49	0·0451	—
C	2·7587	1·30	0·0439	—
		Mean	0·0447	0·0439*

* The discrepancy here is probably due to the fact that a less accurate burette and a stronger solution of $KMnO_4$ were used.

Conclusions and Summary.

1. Iron is estimated in an ashed biological product by means of $N/80$ $KMnO_4$.

2. By using the "reductor" described and titrating in the absence of air a very definite end point is obtained.

3. The method is probably a simpler one than that involving the use of titanium chloride.

I have to thank Dr J. F. Spencer for valuable suggestions.

References.

Barcroft (1914). Respiratory Functions of Blood, 5.
Peters (1912). *J. Physiol.* **44**, 131.

CXII. EXPERIMENTAL ALTERATIONS IN THE CALCIUM CONTENT OF HUMAN SERUM AND URINE.

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From the Biochemical Laboratory, Cambridge.

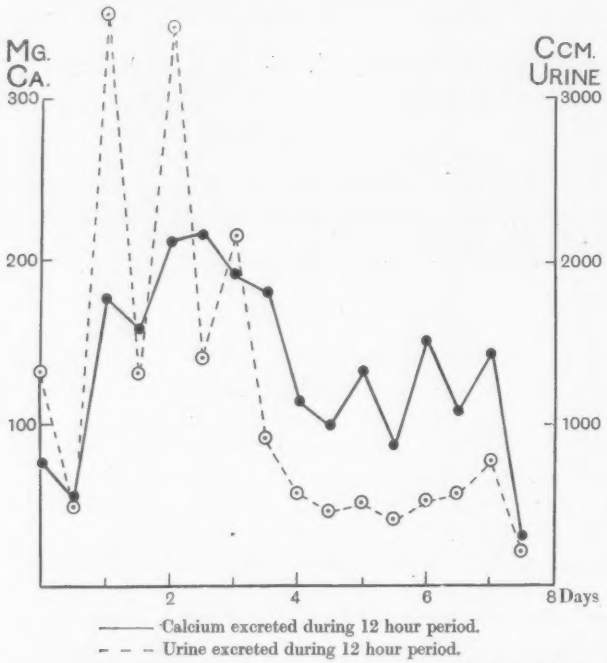
(*Received July 18th, 1924.*)

The most successful attempts to vary the calcium content of normal human serum seem to have been those of Blum, Aubel and Hausknecht [1921], who obtained rises of up to 13 % by the oral administration of calcium chloride, Grant and Goldman [1920] who obtained rises of from 4·4 to 11·9 % by forced breathing, and György [1922], who obtained still larger rises on spasmophilic children with ammonium chloride. In our own experiments, which were all carried out on J.B.S.H. (103 kilograms), we confirmed the above results, and also employed other methods for varying the calcium of the serum.

For serum estimations we used the method of Kramer and Tisdall [1921], the blood being allowed to clot by standing over-night (*i.e.* 18 hours) in a closed tube. All determinations were done in duplicate, and the maximum difference was only 2 % of the amount of calcium found. A few magnesium determinations in serum were also made by the same authors' method. For calcium in urine, the method of Shohl and Pedley [1922] was used.

The results obtained on the calcium and magnesium of the serum are summarised in the Table. In the last experiment with $NaHCO_3$ the deter-

Experiment	Time of drawing blood	mg. Ca per 100 cc. serum	mg. Mg per 100 cc. serum
Normal	—	9·5, 9·5, 9·6	2·48, 2·51
12.30–6.0 p.m. 30 g. $CaCl_2$ ingested	2.30 p.m.	11·7	—
	6.30 p.m.	11·9	2·51
	10·0 a.m. next day	11·1	—
90 minutes forced breathing	End of period	11·4	2·50
90 minutes breathing 6–7 % CO_2	,, ,,	11·1	—
90 minutes breathing 6–7 % CO_2	,, ,,	11·7	2·48
25 g. NH_4Cl ingested	Later in day	10·45	2·56
Day 1; 15 g. NH_4Cl ingested	Day 2	10·5	—
Day 2; 25 g. NH_4Cl ingested	,, 3	10·65	—
Day 3; 25 g. NH_4Cl ingested	,, 5	9·4	—
11.30 a.m.–2.30 p.m. 60 g. $NaHCO_3$ ingested	3 p.m.	9·8	—
	6 p.m.	8·7	2·36
8.30 a.m.–3.0 p.m. 60 g. $NaHCO_3$ ingested	3 p.m.	8·35	—
	5 p.m.	7·5	—
Overnight 30 g. $NaHCO_3$ ingested; 10 a.m.–2 p.m. 60 g. $NaHCO_3$ ingested	4.30 p.m.	8·1 (6·2 "active")	—
12.30–1.15 p.m. 25 g. $MgCl_2$ ingested	3.0 p.m.	9·5	—
	6·0 p.m.	9·7	2·60



Urines were collected at 9.30 a.m. and p.m., the first sample being from 9.30 a.m.–9.30 p.m. 25 g. NH_4Cl were taken on days 1 and 2, 15 g. on day 3, and 15 g. $NaH_2PO_4.H_2O$ on the evening of day 7.

mination was very kindly made by Dr H. W. C. Vines by his method [1921]. The urinary output of calcium varied between 127 and 178 mg. on seven normal days, the mean being 156. After $CaCl_2$ ingestion Haldane, Hill and Luck [1923] found that it might exceed a gram per day. The effects of NH_4Cl are shown in the figure. $NaHCO_3$ produced no definite effect. On a day in which 90 g. were taken 201 mg. of calcium were excreted, but this small rise was perhaps due to a doubling of the urinary volume. The ingestion of 90 g. of hydrated sodium acetate, which was largely oxidised to bicarbonate, giving an alkaline urine containing acetone bodies but no increased diuresis, led to the excretion of only 103 mg.

Discussion.

The changes in the magnesium of the serum are hardly significant. The increased calcium excretion caused by NH_4Cl resembles the increased sodium and potassium excretion described by Haldane, Wigglesworth and Woodrow [1924] in the same experiment, and is presumably due to demineralisation of the tissues. Changes in the calcium content of the serum may be due to at

least two causes. As most of the calcium is associated with colloids and indiffusible, a change in the colloid content of the serum will generally cause a change of calcium content in the same sense. The amount of free calcium may also vary, that associated with the colloids varying in the same sense. Such facts may explain the unexpected agreement between the effects of breathing CO_2 and of overbreathing. The effect of NH_4Cl is probably due at least in part to a concentration of serum colloids parallel with that of the haemoglobin observed by Baird, Douglas, Haldane and Priestley [1923], though presumably the calcium leaving the bones and other tissues must contribute to this increase. With $CaCl_2$, which causes an acidosis like that of NH_4Cl, the observed increase is about half accounted for by this fact, the remainder being due to the calcium absorbed. The main interest of the experiments is that the calcium content of the serum can very easily be altered by a number of methods; but the causes of the alterations are by no means clear.

Summary.

The calcium content of human serum can be raised by 10 to 20 % by ingestion of calcium or ammonium chloride, by breathing 6 to 7 % carbon dioxide, or by forced breathing. It can be lowered by 10 to 20 % by ingestion of sodium bicarbonate.

The urinary calcium output is increased by ingestion of ammonium chloride, little affected by sodium bicarbonate.

The thanks of one of us (C. P. S.) are due to the Trustees of the Carnegie Fund for a grant which made possible participation in this work.

References.

Baird, Douglas, Haldane and Priestley (1923). *J. Physiol.* **57**; *Proc.* xii.
Blum, Aubel and Hausknecht (1921). *Compt. Rend. Soc. Biol.* **85**, 1159.
Grant and Goldman (1920). *Amer. J. Physiol.* **52**, 508.
György (1922). *Jahrb. Kinderheilk.*, **49**, 1.
Haldane, J. B. S., Hill and Luck (1923). *J. Physiol.* **57**, 301.
Haldane, J. B. S., Wigglesworth and Woodrow (1924). *Proc. Roy. Soc. London*, B, **96**, 1.
Kramer and Tisdall (1921). *J. Biol. Chem.* **48**, 223.
Shohl and Pedley (1922). *J. Biol. Chem.* **50**, 537.
Vines (1921). *J. Physiol.* **55**, 86.

CXIII. THE ACTION OF NITROUS ACID UPON THE ANTINEURITIC SUBSTANCE IN YEAST.

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(*Received July 17th, 1924.*)

In the search for the antineuritic substance in yeast, many have abandoned the curative test upon pigeons as a guide in concentration [McCollum and Simmonds, 1918; Seidell, 1922]. In place of the curative test there have been used either (A) experiments involving weight or growth tests, or (B) experiments involving the protection of pigeons from symptoms of polyneuritis while upon a polished rice diet. Procedure (A) suffers from the objection that it assumes at once identity of the curative and growth substances. With (B), on the other hand, difficulties are raised by the variability of the times of onset of convulsions of different pigeons placed upon the same diet. If a given bird therefore upon a given diet does not get symptoms in a given time, it is not necessarily a clear proof of the activity of an experimental addition to the diet, because there is always the possibility that the bird in question might not have become polyneuritic in the time taken even upon polished rice alone. Further there is a call for comparatively large amounts of experimental material which inhibits rapid progress.

Considerations of this nature led me to adhere solely to the problem of concentration of the *curative* substance or substances in yeast extracts. An experience of over two years of the behaviour of pigeons fed upon polished rice has convinced me that curative tests can be used as a guide in concentration under stated conditions.

I have thought it worth while to enumerate these, not because they establish anything new, but because it is difficult to find such information collected.

The Curative Test.

1. *Symptoms.* Out of a given batch of pigeons fed upon polished rice, a majority show the classical symptom of head retraction. A few cases show the emprosthotonos of McCarrison [1919], while the remainder merely become progressively lame and die suddenly, usually without convulsions. It is the safest course to work with those showing the classical symptoms of head retraction, and to discard the other cases [Williams, R. R., 1917]. The cases of lameness often cannot be cured with marmite. It is a good plan to make a rule that any pigeon which has not reached the typical condition of head retraction within 30–35 days be put back upon the normal diet for a few weeks, and then returned to the polished rice.

2. *Rice feeding.* Confining ourselves to the pigeons showing the classical symptom, birds seem to get symptoms more rapidly when they are allowed to fly about outside in a large open cage. This was discovered by chance owing to the difficulty of finding accommodation for a large batch of pigeons indoors. A number of the batch were placed outside in the cool weather, the remainder being placed in cages in a warm room. To my surprise some 40 % of the pigeons outside showed symptoms in 11–30 days whereas the pigeons inside did not become polyneuritic until after this time. I have made no attempt to investigate this matter, which is possibly related to the increased metabolism. It is a curious fact that pigeons with threatening convulsions will often recover for a few days when brought into the warm room after being outside, and in conversation Col. McCarrison has told me that he has noticed the precipitating effect of cold upon the convulsions. In view of some recent remarks by Blacklock [1924], in which he states that lack of exercise is a contributory cause in the acceleration of death in polished rice pigeons, it may be that cold is the predominating factor in the acceleration of symptoms among the pigeons placed outside. As a practical point I have adopted the plan of keeping the pigeons at the beginning of their polished rice diet in an outdoor shed, in which they can fly about. When they show symptoms of weakness and are unable to fly from the ground to their perch, they are brought into cages in a warm room, where they remain until the symptoms appear. As in most cases of animals upon deficient diets, it is important in the final stages to avoid cold shock to which the birds seem to be especially liable. When treated in the above way, a good number of cases are obtained without recourse to artificial feeding and as the symptoms are reached soon, the birds have the necessary strength to weather the tests satisfactorily.

3. *The test.* The real difficulty in the use of the curative test lies in the interpretation of the effects of administration of the curative substance. The difficulties are twofold. Firstly, many substances other than the one sought for may produce temporary cures. Secondly, pigeons may rapidly get into a condition in which not even an accepted curative extract such as marmite will produce recovery. I shall deal with these two conditions in turn.

1. *Temporary improvement.* In some cases a dose of distilled water even may induce an amelioration of symptoms. The following experiment 1, illustrates this point.

Exp. 1. 11 Feb. Pigeon showing slight symptoms of head retraction and lameness.
1 p.m. 20 cc. of distilled water given by mouth.
6 p.m. Distinctly better. Polished rice given.
12 Feb. 12 noon. Symptoms worse again. Given marmite.
4 p.m. Looked normal.

This is a case of temporary improvement for 24 hours in which the pigeon has been able to right its physiological instability for a short time with the help of the water. There are many recorded cases of temporary improvement

following the injection or giving by the mouth of diverse substances, a case in point being the series of purine compounds tested by Funk [1913]. I have come to the general conclusion that a curative experiment cannot be considered decisive unless (*a*) the symptoms ameliorate considerably within 3–6 hours, and (*b*) the cure persists for over 48 hours, while the bird is still upon a polished rice diet. It is further more satisfactory if symptoms can be cleared up more than once, though this has certain limitations. Upon a second or third appearance of the convulsions the condition seem to be more rapidly progressive than upon the first appearance. This imposes the limitation that the curative substance be administered within an hour or so of the second onset of symptoms. Where this has been done, it has been found possible in good cases to clear up symptoms as many as four times. Exp. 2 illustrates this.

Exp. 2. 21 April. Head retraction.

7.20 p.m. Curative dose. Rather better in 3 hours. Cured next morning.

23 April, 12 noon. Symptoms again, treated within an hour with more curative extract. Improved in 6 hours and well next day.

28 April, 9.15 a.m. Symptoms again. More extract. Well next day.

6 May, 12 noon. Symptoms again. More curative extract. Well next day.

8 May. Placed upon normal diet again.

2. *Intractable cases*, in which even marmite will not produce a cure. Comparatively often I have been unable to cure pigeons by feeding them upon marmite. Among a series of over 200 cases I find that I have tried to clear up some 59 with marmite at various stages after the onset of symptoms, and usually after attempts to cure with other substances. An analysis of these cases shows the results in Table I.

Table I.

	Number of hours after the onset of symptoms	
	24	48
Not cured	11	10
Cured	31	7

Out of 42 pigeons, therefore, attempts to clear up within 24 hours gave 31 cures, some 75 %, whereas the proportion is much less (about 40 %) for birds allowed to continue longer. Not much stress is laid upon these figures, but they show the order of things which can be expected. The practical conclusion to be drawn is that a failure to produce cure by a test substance administered after long continuance of the symptoms should not be accepted as proof of the absence of the curative substance from the extract. We may summarise the conditions for the curative test in somewhat the following way:

1. Only those pigeons should be used for the test which show well-marked head retraction, in which the symptoms have appeared within 30–35 days, and which appear strong enough to withstand treatment.

2. If the symptoms clear up completely upon administration of the extract within a period of 6–12 hours, recovery must be a persistent one for two or better three days, in order to establish the activity of an extract.

3. If the symptoms do not clear up, it is not proof of the non-activity of an extract unless (*a*) the bird is strong, and (*b*) the extract is administered within 12 hours from the start of the convulsions if it is the first attack, and within three hours if it is the second.

Extracts have been administered by passing directly into the crop by the use of a pipette fitted with a small piece of bicycle valve tubing. Care must be taken to see that the bird does not drown itself when there is spasm of the throat, leading to passage of the administered solution into the trachea, and so to the lungs.

Histamine not the curative substance.

The asserted presence of histamine in yeast extracts [Seidell, 1921], together with the claim made by Danysz, Michel and Koskowski [1922] and by Koskowski [1922] that pigeons could be protected from convulsions upon a polished rice diet by injections of histamine, led me early in the work to test the curative effect of this substance. The results of the experiments confirm in the main the conclusions of Abderhalden [1923] that definite cures can be produced, but that they are quantitatively and usually qualitatively different from those produced by yeast extracts.

I have made curative experiments both by feeding and injection, and protective feeding experiments, using a B.D.H. specimen of histamine phosphate.

Curative experiments.

Exp. 3. *Injection experiments.* In three experiments in which varying amounts of histamine were injected no effect was observed. This confirms the results of Danysz, Michel and Koskowski [1922].

Exp. 4. *Feeding by the mouth.* The administration of histamine in doses of approximately 0·2 mg. up to a maximum of 0·6–1·0 mg. gave the following results. Out of 18 birds treated, not improved 6, improved for periods up to 24 hours 4, and up to 48 hours 3. The remaining 5 were cured for 3, 5, 6, 8, 12 days respectively. Accepting the criteria laid down in the earlier part of this paper, 28 % would be regarded as cured. It is clear, however, that we are not dealing with the certainty of a yeast extract with which, under comparatively unfavourable conditions, a 75 % cure was obtained. The difference of the histamine effect from that of the curative substance in yeast extracts is also made clear by Exp. 5, in which daily feeding of histamine in 0·4 mg. doses did not influence the onset of convulsions.

Exp. 5. Two batches of 10 pigeons were kept under the same experimental conditions outside in an outdoor cage, merely being separated by a wire partition. One batch received 0·4 mg. of histamine by the mouth each day after the second. Up to the thirty-first day the following were the days after starting the feeding upon which the pigeons showed polyneuritis.

Day upon which convulsions appeared:

Histamine fed 12, 15, 21, 25, 25, 31, 31.
No histamine 19, 23, 26, 26, 26.

Exp. 5 shows that there was nothing to choose between the times of onset of convulsions for the two sets of birds. Moreover, none of these cases could be cleared up by giving more histamine. Presence of vitamine contamination in the preparation would seem to be excluded by experiment, so that we must attribute the occasional curative effect of histamine by the mouth to some other obscure cause, possibly a family relation to the actual curative substance.

Certain other substances.

A variety of other substances were tried in an attempt to see whether the effect of histamine was related in any way to its basic character. Table II shows that all the substances tried were definitely less effective than histamine, a result which has also been reported by Abderhalden for amines excluding tyramine. Out of the 22 pigeons treated with various substances, only one case could be ranked as a cure, a different picture from histamine. The failure of effect of sodium bicarbonate shows that the onset of symptoms is not due to an upset of the alkalinity of the blood owing to discharge of acid into the blood. This was suggested by the ease with which exercise precipitates the convulsive condition in these birds.

Table II.

Substance	Dose	No. of birds	Effect
Na bicarbonate	0·5 g.	5	Nil in three cases. Temporary improvement for 10 hours and 30 hours in two cases
Hexylamine	20 mg. (approx.)	6	Nil in two cases. Temporary improvement for 24 hours in two cases; 2 days in two cases
Guanidine	7 mg.	1	No effect
Dimethyl urea	9 mg.	1	Improvement for 24 hours
Urea	0·05 g.	2	Nil in one case. Slight improvement for 4 hours in other case
Olive oil	4–7 cc.	5	Nil in one case. Improved for 12 hours, 30 hours, 45 hours (three cases). One case cured for 6 days

That the curative effect of a yeast extract is not due to primary amines is, I think, definitely shown by the experiments with nitrous acid described later in the paper. Before turning to these, however, a convenient method of obtaining concentrated extracts of yeast will be described in which use is made of a charcoal method of extraction.

Preparation of concentrates of the curative substance in yeast.

Yeast in 7 lb. samples is kept at room temperature for two days. It is then boiled up with tap water twice, approximately 1500 cc. being used for each of the boilings [Osborne and Wakeman, 1919]. To the combined filtrates, 25 % neutral lead acetate is added in an amount just short of that required to produce the maximum precipitate. This is usually slightly more than 300 cc. The filtrate from this is made definitely acid with sulphuric acid and treated with the acid mercuric sulphate reagent (Hopkins), until the maximum

precipitation is obtained. It is allowed to stand overnight and then filtered. The clear filtrate is allowed to stand for a day in the course of which time it usually deposits a faint cloud. This should be removed by filtration. To the clear filtrate some 10 g. of BaS is added, and the mixture is filtered clear. If this should prove difficult at this stage, a cautious addition of $Ba(OH)_2$ will usually make it possible to reach a stage at which a clear filtrate is obtained. The fluid, however, should not be allowed to get alkaline, and is usually of a high acidity when the filtering point is reached. The fluid cleared of the sulphides and $BaSO_4$ is boiled to expel H_2S and cooled. It is then exactly neutralised with NaOH to litmus, and made just acid with acetic acid, any precipitate which forms being removed by filtration. 30 g. of "norite" charcoal is then stirred into the fluid, and allowed to settle for 30 minutes. The charcoal is separated upon a Buchner funnel, and washed thoroughly with distilled water. If the filtrate from the charcoal is still coloured it is treated with 20 g. more charcoal, and this is then combined with the original charcoal fraction.

The charcoal is treated with 300 cc. of 50 % alcohol containing 1 cc. of strong HCl per 100 cc. The mixture is heated for half an hour upon the water bath and filtered. The charcoal is again extracted with two successive 150 cc. amounts of alcohol of the same strength and acidity. The alcoholic extract is evaporated *in vacuo* at a temperature not exceeding 60°, to a small bulk of about 20 cc.

Several extracts of yeast have been made in this way and have all proved highly curative. An amount of the order of 0·05 cc. will cure the usual pigeon of symptoms within 6 hours and keep it from convulsions upon a diet of polished rice for a matter of $3\frac{1}{2}$ days. Such an amount after drying at 100° will contain about 10·5 mg. of solid of which 1·2 mg. is ash, so that under 3 mg. is needed as a daily dose. This compares favourably with other concentrates. Provided that the HCl present in these concentrates is not removed they will keep for a period of several months at room temperature. This amply confirms the remarks upon stability in the presence of acid made by other observers (Seidell).

The action of nitrous acid.

So far as I know there is no experiment in the literature upon the action of nitrous acid upon the curative effect of yeast extracts. McCollum and Simmonds [1918] showed that the growth substance for rats was not much affected by treatment for four hours with nitrous acid gas. In my experiments (Table III), treatment with an acid solution of sodium nitrite did not destroy the curative action of the yeast concentrates prepared with charcoal. Exps. 6, 7, 8 (Table III) shows that it is possible to get roughly quantitative results with these extracts in terms of the time after cure for which protection lasts upon the polished rice diet. Exps. 9, 10, 11, 12 (Table III) represent the effects of part of the same yeast concentrate treated with nitrite before administra-

tion. The nitrite treatment has been varied in different experiments without effect upon the results obtained. The usual procedure has been to add saturated sodium nitrite drop by drop to the acid yeast concentrate either at room temperature or in ice water, allowing the action to proceed for 12 hours or more. The mixture is then warmed for a short time and finally boiled, cooled and neutralised before administration to the pigeon. In all cases there has been no appreciable destruction of the curative substance so far as can be judged from the rough quantitative results obtainable.

Table III.

Exp.	Amount given	Improvement in	Protection for over
	Yeast concentrate before nitrite treatment.		
6	1 cc.	5 hours	16 days*
7	$\frac{1}{2}$ „	4 „	9 „
8	$\frac{1}{12}$ „	4 „	4 „
	Yeast concentrate after nitrite treatment.		
9	$\frac{1}{12}$ cc.	5 hours	5 days
10	$\frac{1}{12}$ „	7 „	6 „
11	$\frac{1}{12}$ „	4 „	10 „
12	$\frac{1}{12}$ „	6 „	7 „

* Bird then placed on normal diet.

The curative activity of the concentrates after treatment with nitrous acid eliminates not only histamine but probably all primary amines as being the curative substance. A large number of secondary amines are also excluded, though certain of the more complicated ring structures containing the :NH grouping will probably survive the treatment mentioned. In the case of certain nitrosamines, reconversion to the secondary amine by heating with HCl is known to be readily brought about.

There is still left the puzzle as to the action of histamine in certain cases. Upon the whole I feel tempted to view the symptoms as an expression of some complex local vascular disturbance and to attribute the effect of histamine to some temporary vascular action, but any such view only adds one more to the speculations which have already been advanced in connection with the antineuritic vitamin.

Conclusions.

1. The curative test upon the polyneuritic pigeon can be used as a guide in concentration experiments under stated conditions.

2. In confirmation of Abderhalden, the administration of histamine as a curative agent sometimes produces cures in pigeons. Daily doses of histamine do not delay the onset of symptoms.

3. Treatment of acid extracts of yeast with nitrite does not destroy the curative property of such extracts. This excludes primary amines and many secondary amines as responsible for antineuritic action.

I am indebted to Prof. Hopkins for encouragement and the facilities offered for this work while at Cambridge, also to Miss Kilby and H. Mowl for help with the pigeons. I am also grateful to H. Poulter for his help in the preparation of yeast concentrates.

REFERENCES.

(For references to the vast literature see the Report of the Medical Research Committee on the present state of knowledge of accessory food factors, 2nd ed. 1924, *Med. Res. Council Special Rep. Series*, No. 38 (revised).)

Abderhalden (1923). *Pflüger's Arch.* **198**, 570.
Blacklock (1924). *Brit. Med. J.* i, 1046.
Danysz, Michel and Koskowski (1922). *Compt. Rend. Acad. Sci.* **175**, 54.
Funk (1913). *J. Physiol.* **45**, 489.
Koskowski (1922). *Arch. int. Pharm. Ther.* **26**, 359.
McCarrison (1919). *Ind. J. Med. Res.* **6**, 275.
McCollum and Simmonds (1918). *J. Biol. Chem.* **33**, 55.
Osborne and Wakeman (1919). *J. Biol. Chem.* **40**, 383.
Seidell (1921). *J. Ind. Eng. Chem.* **13**, 1114.
—— (1922). *J. Amer. Chem. Soc.* **44**, 2042.
Williams, R. R. (1917). *J. Biol. Chem.* **29**, 504.

CXIV. ALLELOCATALYSIS AND THE GROWTH OF YEAST.

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(*Received July 18th, 1924.*)

Brailsford Robertson [1921] has claimed that in the case of certain infusoria, if two individuals are isolated into a drop of culture medium the growth produced is in a given time far more than double that produced by a single cell under similar conditions. He claims further that this phenomenon, to which he has given the name "allelocatalysis," only occurs provided that the organism is supplied with a substance, termed by him X, which is produced in media as a result of bacterial metabolism and accelerates the growth of single cell cultures of the organism. His conclusions have been based on experiments in which isolated *Enchelys* have been grown in media containing bacteria, and I can find no records in his experiments of any bacteria-free cultures. Cutler and Crump [1923], working with a culture of *Colpidium* which contained only one contaminating organism, were unable to corroborate Robertson's claims.

In the following communication I have recorded the results of some experiments in which a pure culture of yeast has been grown free from bacterial contamination in hanging-drop preparations containing one, two, three, and in a few cases five cells. The growth has, moreover, been studied in media containing various concentrations of "bios." The conditions therefore have been strictly analogous to those under which Robertson has observed the occurrence of allelocatalysis, except that the bacterial factor has been eliminated. Experiments are at present in progress to determine how far the growth of yeast may be influenced by the presence of bacteria.

Experimental.

General procedure.

For convenience a basal medium was prepared which had the following composition:

Basal Medium.

Primary potassium phosphate (KH_2PO_4)	5·00 g.
Ammonium chloride	2·50 g.
Magnesium sulphate	0·35 g.
Calcium chloride	0·25 g.
Glass-distilled water to 1000 cc.	

The media used in these experiments were prepared from the basal medium as follows:

Medium 1. Basal medium + 50 g. per litre commercial cane sugar.

Medium 2. Basal medium + 50 g. per litre recrystallised cane sugar.

Medium 3. Basal medium + 50 g. per litre recrystallised cane sugar, to which was added one quarter of its volume of a solution containing 12·0 mg. per 100 cc. of a solid "bios complex."

The recrystallised cane sugar was prepared from a commercial sample by three crystallisations from hot 80 % alcohol, according to the method of Willaman and Olsen [1923].

The "bios complex" was prepared according to the method of Eddy, Kerr and Williams to whom I am indebted for their courtesy in sending me details of the method previous to its publication.

Unless otherwise stated distilled water was obtained by distilling tap-water from alkaline permanganate in an all-glass apparatus, and collecting the middle portion of distillate. The first portion was used for rinsing glass-ware, etc., previous to sterilisation.

The salts used were Kahlbaum's "Zur Analyse" and with the exception of the calcium chloride were recrystallised twice from the glass-distilled water.

All glassware was stood for at least twelve hours under a mixture of nitric and chromic acids, rinsed first in running tap-water, then ordinary distilled and finally in glass-distilled water. It was then dried at 110° before use, with the exception of cover slips, which were dried by rinsing in 70 % alcohol (containing 0·5 % HCl) and wiping with a clean dry cloth. As far as possible the glassware used was Jena or Pyrex. All glassware was sterilised by dry heat at 170°–180° for twenty minutes. For sterilisation cover slips were put up in deep Petrie dishes (3 inches by 1½ inches), and depression slides in plugged boiling tubes. The media were sterilised immediately they were made up by steam heat in a Koch steriliser on three successive days. The vaseline used throughout these experiments was sterilised by heating to 170°–180° for twenty minutes. Silica pipettes, which could be sterilised by heating to redness in a flame, were found useful for many of the operations [Peters, 1921].

The culture used was a pure culture of *Saccharomyces cerevisiae* obtained from the Lister Institute (Nat. Type Culture 381/21). The stock culture was grown in 5 cc. portions of Medium 1 unless otherwise stated and repeatedly subcultured (about every three days). All operations involving the exposure of sterile cultures were conducted with the usual precautions against bacterial contamination in a cupboard fitted with glass back and front, which was steamed for about half an hour on the day before use, and remained closed until the time of experiment.

Method.

The procedure in each experiment was as follows. It is based on the method used by R. J. Williams [1919]. From the culture selected for growth a

sample was removed, taking care to obtain as many yeast cells as possible from the bottom of the tube in a minimum volume of medium. This sample was immediately placed in a test-tube, and may be called, for convenience, the "parent culture." Having placed in readiness a depression slide ringed with vaseline, inverted and held in a clip over a cover slip, the "parent culture" was shaken thoroughly and a small sample removed in a capillary pipette. This sample was then immediately placed out in drops on the cover slip, and the depression slide brought down carefully so as to pick up the cover slip without touching the cultures with vaseline. The slide was then inverted and the edges of the cover slip gently pressed down to make an air-tight ring of vaseline. In the later experiments a platinum nib, mounted in a glass rod, was found more convenient than a capillary pipette for placing out the culture in drops.

By a count of the average number of cells in these drops of "parent culture," and by appropriate dilution of another sample of the "parent" with the required medium, a tube of culture was made up from which a series of hanging drop preparations were made containing the required number of cells per drop. To diminish the chances of contamination it was usual to make up a fresh tube of culture for every four or five slides.

It was usual to put up at the same time one slide containing drops of medium which had been lightly infected from a very mixed bacterial culture. This was grown as a control at 25° to show the degree of bacterial growth to be expected in any accidentally infected culture, and to make sure that the technique of drying and staining the cultures was adequate to show up bacteria.

After a little practice it was found that the maximum time during which the cover slip was exposed did not exceed thirty seconds. All the operations were, moreover, conducted in the aseptic cupboard as far away from the sterilising burner as possible. By these means the risk of infection or evaporation of the cultures was minimised, and it was actually found that only a very small proportion of the cultures had to be discarded on account of the presence of bacteria.

Each slide was examined under the microscope immediately after it was put up, and the position of each drop which contained cells was recorded, together with the number of cells it contained. Buds of less than half the diameter of an average sized yeast cell were not counted, those of which the diameter was more than half that of an average sized cell were reckoned as cells. The slide was then placed in the thermostat at 25° and the time noted. At various definite intervals after the time of incubation the slides were examined and growth recorded, and during the course of the second day of growth the cultures were examined under the high power (magnification × 700) and the absence of bacteria confirmed by observation.

As an approximate estimate of the drop volumes the diameter of the drops was measured and recorded. Most of the cultures in each experiment were in

drops of the same size. In a few cases, however, there were marked differences in size, but in none of these did there appear to be any marked changes in the growth. Experiments are in progress to determine whether there is any influence of drop volume on the growth of drop cultures, as the subject is of some importance in connection with the problem of "bios" and Pasteur's original observation.

At the conclusion of the experiment each cover slip was treated as follows. The position of the cultures was marked temporarily by means of Indian ink. The cover slip was carefully removed from its slide and permanent marks etched on it by means of hydrofluoric acid to establish the identity of each culture. The drops were dried off *in situ* over phosphoric anhydride *in vacuo* at 40°. The cover slips were now treated as bacteriological smears, i.e. they were fixed, stained by the Claudius' modification of Gram's technique, and mounted for bacteriological examination under an oil immersion objective. By this means it was possible to establish with certainty the presence or absence of bacteria.

Results.

In Table I, I have recorded the results obtained in four experiments. The only cultures in these experiments which have been omitted from the table are three in which the growth was so extremely slow that it had not commenced until after the thirtieth hour. All the cultures were free from bacterial contamination according to the tests described above, and no culture has been omitted on account of exceptionally high growth.

Table I.

Exp. 1. Medium 2

Number of cells present after 40 hours at 25° in cultures containing originally

1 cell	2 cells (A)	2 cells (B) 1 growing?	3 cells (B) 1 growing?	5 cells (B) 2 growing?
9, 17, 16, 11, 16, 19, 18 (15·1)	22, 32, 28 (27·3)	8, 8, 14, 9 (9·8)	13	32
The corresponding drop diameters were, in millimetres				
0·9, 0·8, 0·9, 0·6, 1·0, 0·9, 0·8 (0·84)	0·9, 1·0, 1·0 (0·97)	0·7, 0·7, 1·0, 0·8 (0·80)	0·8	1·0

Exp. 2. Medium 1

Number of cells present after 32 hours at 25° in cultures containing originally

1 cell	2 cells (A)	3 cells (B) 2 growing?	4 cells (B) 2 growing?
4, 5	7, 8	7	6, 5, 8 (6·3)
The corresponding drop diameters were, in millimetres			
0·4, 0·5	0·6, 0·4	0·4	1·0, 0·6, 0·5

Exp. 3. Medium 1

Number of cells present after 18 hours at 25° in cultures containing originally

1 cell	2 cells (A)	3 cells (A)	5 cells (A)	3 cells (B) 1 growing?
28, 30, 27 (28·3)	70, 89	95	125	31, 37, 27, 45 (35·0)
The drop diameters were not measured.				

Exp. 4. Medium 3

Number of cells present after 24 hours at 25° in cultures containing originally				
1 cell	2 cells (A)	3 cells (A)	2 cells (B) 1 growing?	3 cells (B) 2 growing?
7, 18, 8	17, 22	28, 47	7, 8, 5	16, 13, 15
The corresponding drop diameters were, in millimetres				
2·1, 1·7, 2·2	2·0, 2·2	3·0, 1·9	2·0, 2·0, 2·1	1·9, 2·0, 2·6

It will be seen that in those cultures which contained more than one cell I have in many instances divided the results into two groups. In one group (A) the results are of such an order as to indicate that all the cells originally present have participated in the growth produced. In the other group (B) this is obviously not the case, and I have in these cases entered at the head of the column the number of cells which can, I think, be assumed to be responsible for the growth. The figures in brackets indicate averages of the figures below which they are placed in the table.

In Exp. 1 it will be seen that there is no evidence of any growth which might be considered allelocatalytic. The maximum growth produced by a single cell was 19, that of two cells 32, that is, less than double that of one cell.

In Exps. 2 and 3 although the medium was the same in both experiments the growth produced was very different. Possibly the small size of the drops in Exp. 2 was responsible for the slow growth. The cells in Exp. 3 were washed with Medium 2 previous to being suspended in Medium 1 in a tube closed with a cotton-wool plug in which the plug was used as a filter which did not allow many cells to pass. Possibly some growth factor was absorbed from the wool. The experiments have been recorded in view of the fact that in spite of the extremely slow growth in the one case and the extremely rapid growth in the other, there was again little evidence of allelocatalysis. The figures 70, 89, 95, 125 are subject to error in that the colonies tend to grow in three dimensions, and any number of cells greater than 50 is somewhat difficult to count with accuracy.

In Exp. 4 the maximum growth of one cell was 18, two cells 22, three cells 47. The stock culture used in this experiment had been grown in Medium 3.

In four other similar experiments, comprising 41 bacteria-free cultures, there was only one culture in which two cells produced more than double the number produced by one cell in the same time. Bearing in mind that in those cases where evidence of allelocatalysis has been observed it is possible that other cells have been present originally but have been missed in the initial count, the following conclusion seems justified:

In the case of the yeast studied the growth of two cells is, within the limits of experimental error, approximately double that of one cell provided, of course, that the conditions of growth are the same. The growth of a given yeast cell is not therefore accelerated by the presence of other living yeast cells.

If the assumption as to the number of cells which were growing in the various cultures under group B has been correct, we should also expect to

find that the growth of a single cell is not accelerated by the contiguity of other dead yeast cells. In Table II I have recorded the results obtained in a few bacteria-free cultures in which I have been fortunate to observe the growth of one cell in the presence of other cells which did not themselves grow.

Table II.

Exp. 5. Medium 3

Number of cells present after 24 hours at 25° in cultures originally containing	
1 cell	1 cell in the presence of 2 others which did not grow
7, 18, 8	8, 7
The corresponding drop diameters were, in millimetres	
2·1, 1·7, 2·2	2·0, 2·1

Exp. 6. Medium 2

Number of cells present after 48 hours at 25° in cultures originally containing	
1 cell	1 cell in the presence of 4 others which did not grow
7, 16	5, 23, 7
The corresponding drop diameters were, in millimetres	
0·5, 0·5	0·5, 0·6, 0·6

In Exps. 5 and 6 it will be seen that there is only one case in which the growth of one cell is accelerated by the presence of other dead cells. In two cultures in another experiment I have studied the growth of two cells in the presence of other dead cells, and there was no evidence of acceleration. The following conclusion is therefore justified.

Conclusion.

The growth of bacteria-free cultures of yeast in hanging-drop preparations containing one, two (or three) cells is proportional to the number of cells present, provided that all the cells are living. The presence of dead cells does not accelerate the growth. Allelocatalysis does not occur in the case of the yeast studied.

In conclusion I wish to thank Professor Peters for his kind interest and advice throughout the research, and Mr Carleton for much valuable advice in elaborating the technique adopted for the bacteriological tests.

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REFERENCES.

Cutler and Crump (1923). *Biochem. J.* **17**, 878.
Peters (1921). *J. Physiol.* **55**, 1.
Robertson (1921). *Biochem. J.* **15**, 612.
Willaman and Olsen (1923). *J. Biol. Chem.* **55**, 815.
Williams, R. J. (1919). *J. Biol. Chem.* **38**, 465.

CXV. THE RELATION OF THE FAT-SOLUBLE FACTOR TO RICKETS AND GROWTH IN PIGS. III.

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WITH A REPORT ON THE PATHOLOGICAL HISTOLOGY OF THE BONES, BY V. KORENCHEVSKY.

From the Dept. of Experimental Pathology, Lister Institute.

(*Received July 21st, 1924.*)

Some years ago we began this investigation with the object of studying the possibility of inducing rickets in pigs by dietetic means. We have already shown [Zilva, Golding, Drummond and Coward, 1921; Golding, Zilva, Drummond and Coward, 1922] that by depriving the animals of vitamin A growth was appreciably restricted, and was accompanied by osteoporosis. The histological examination of the ribs of such animals, which was kindly undertaken for us by Prof. Korenchevsky, did not reveal any significant increases of osteoid tissue, a condition which is considered to be pathognomonic of rickets.

In this communication we are bringing forward evidence which we believe demonstrates that under certain conditions it is possible to induce typical rickets in pigs by depriving them of the fat-soluble antirachitic vitamin. The systematic experiments described here were the outcome of an observation that pigs, fed on a diet which included dried separated milk in large quantities and which was otherwise almost free from the fat-soluble vitamins, grew well but eventually displayed rachitic symptoms.

Experimental.

The problem was investigated in two sets of experiments. In one, all the animals were studied in the absence of direct sunlight. The second was a repetition of the first, except that two of the animals were exposed to direct sunlight and given cod-liver oil in addition.

Exp. 1. In this experiment eleven pigs were employed; they consisted of selected Middle Whites and crosses between Berkshires and Middle Whites, and belonged to two litters farrowed on the same day (February 14th, 1923).

The mothers of these litters were kept under normal farm conditions at the time of service and during gestation. At the commencement of the experiment (April 17th, 1923), the pigs were 62 days old. The diet consisted of toppings, barley meal, dried separated milk, "animal charcoal," swedes and chalk. The average daily consumption and calcium and phosphorus content of the foodstuffs were as follows:

	CaO %	P_2O_5 %	Average daily consumption in oz.
Toppings	0·113	2·06	27
Barley meal ...	0·189	0·930	2
Dried separated milk	2·16	2·39	6·5
Animal charcoal ...	40·00	33·1	1
Chalk	50·8	—	1
Swedes	—	—	3

The average intake of CaO per day was, therefore, 1·085 oz., and that of P_2O_5, 1·06 oz., giving a ratio of Ca/P = 1/0·6. The dried separated milk powder was about nine months old and was prepared from a summer milk. It was kindly supplied to us by Messrs J. Lyons and Co., Ltd.

One group of the animals received swedes from April 30th, as an anti-scorbutic. The other group was not given any vitamin C. The animals were arranged as follows:

No.	Sex	Breed	Weight at commencement of experiment in lbs.	
122	Sow	Middle White	30	Received swedes
124	Hog	" "	33	" "
126	"	" "	41	" "
128	"	Berkshire × Middle White	30·5	" "
130	"	" "	31·5	" "
121	"	Middle White	33	Received no swedes
123	"	" "	28·5	" "
125	"	" "	35	" "
127	"	Berkshire × Middle White	39	" "
129	"	" "	36·5	" "
131	"	" "	33	" "

All the pigs were kept in a roomy animal house with gauze blinds drawn and were allowed sufficient exercise. The following is the protocol of the animals:

Pig 122. This animal first showed lameness in all its limbs on June 8th. This condition became gradually worse. On July 9th the administration of cod-liver oil was begun, the basal diet and other conditions being kept the same. The initial dose was $\frac{1}{4}$ oz. of oil per day, which was eventually increased to $\frac{1}{2}$ oz. The animal gradually improved, and by the end of August its condition appeared to be normal. At the same time the rate of growth was greatly increased, the animal gaining at the rate of about 1·25 lbs. per day as against 0·77 lb. a day during the period before the cod-liver oil was given.

Pig. 124. This animal grew at the rate of about 0·7 lb. per day until June 15th when it became very lame. On July 6th its condition was so bad that it was decided to kill it.

Professor Korenchevsky's report upon the histology of the rib junctions in this and the other pigs which came to autopsy is given below, p. 876.

Pig 126. This animal grew at the rate of about 0·7 lb. per day until June 6th, when its growth almost ceased. A few days previously (May 30th), the animal became very lame. This condition became worse and on July 6th it was slaughtered.

Pig 128. This animal gained at the rate of about 0·9 lb. per day. Lameness was first observed on June 8th and gradually became worse, the animal crawling on its knees. It was killed on June 20th.

Figures 8 and 9 portray the condition of the animal before being killed.

Pig 130. Lameness was first observed intermittently from May 3rd until June 29th, after which period the lameness became permanent. Considerable swelling at the joints was observed from June 8th. On July 6th the animal was down on its knees. On July 9th cod-liver oil was administered. The weight curve of the animal is given in Fig. 1. Figs. 2 and 3 portray the appear-

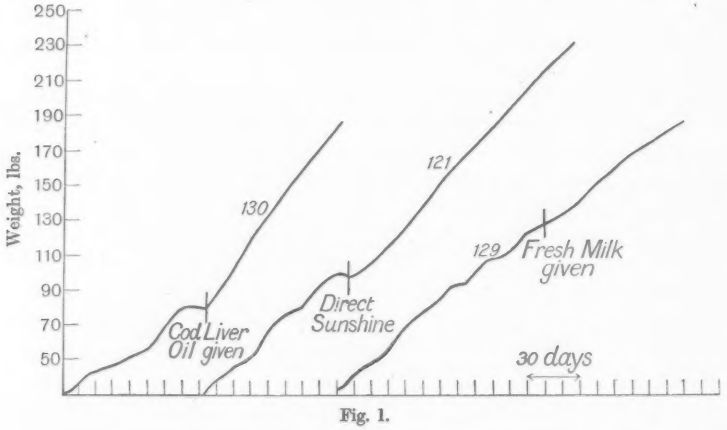

Fig. 1.

ance of the pig before and after the cod-liver oil treatment. It was killed on September 19th and the report of the histological examination of the ribs is given below.

Pig. 121. Slight signs of lameness were observed on June 15th. From this date the condition varied, sometimes the pig appeared to be better, but gradually became worse; the animal was very badly "off its feet" on July 12th. At this stage it was allowed access to direct sunlight for a number of hours daily. The diet remained the same. The condition of the animal improved and the rate of growth increased, although the change was not so marked as in the case of the animal cured by cod-liver oil. It was killed on November 19th. Prof. Korenchevsky reports that the histological examination of the ribs revealed almost a normal condition. The weight curve of this animal is given

in Fig. 1, and Figs. 4 and 5 show its appearance before and after its exposure to direct sunlight.

Pig. 123. The first sign of lameness was observed on June 15th and this gradually developed. On July 12th this animal was exposed to direct sunlight in the same way as Pig 121, and as in the case of that animal the condition of the pig and its rate of growth improved. It was killed on November 19th, and, as will be seen from Prof. Korenchevsky's report below, the histological examination revealed osteoporosis but not rickets.

Pig 125. This animal grew at the rate of about 0·9 lb. per day. It first showed lameness on June 8th, from which date the condition varied until July 26th, when it became very bad and the animal was killed. The report on the histological examination of the ribs is given later.

Pig 127. This pig gained at the rate of about 0·8 lb. daily. First signs of lameness observed on June 8th, and by July 17th, the animal was "off its feet." It was killed on July 30th.

Pig 129. This pig gained at the rate of about 0·8 lb. daily. First signs of lameness observed on July 20th. On August 26th the dried separated milk was replaced by fresh full-cream cow's milk (1 quart per day). As will be seen from Fig. 1, the animal continued to grow at the same rate as before. The symptoms of lameness definitely improved after the treatment, but did not entirely disappear even after 80 days on the fresh milk diet which was evidently not good enough to effect a complete cure. Figs. 6 and 7 portray the appearance of the animal before and after the addition of the fresh full milk to the diet.

Pig 131. This pig gained at the rate of about 0·8 lb. daily. The first signs of lameness were observed on June 15th. This condition varied for some time and the pig was killed on September 19th. For the result of the histological examination of the ribs, see below.

Exp. 2. This experiment was devised for confirmatory purposes with the modification that two animals were exposed to sunlight.

They were arranged as follows:

No.	Sex	Breed	Weight at commencement of experiment in lbs.
133	Sow	Berkshire and Middle White	40
136	Hog	" " "	44½
138	"	" " "	38½
134	Sow	" " "	39
140	Hog	" " "	46
137	"	" " "	33
132	"	" " "	20
135	"	" " "	22½

The animals received the same basal diet as the pigs in Exp. 1. Pigs 134, 140 and 137 were given apples to supply the antiscorbutic factor. Control Pigs 132, 135 received apples and cod-liver oil and were allowed access to a pen outside the stye where they had the benefit of direct sunlight during the day. All the animals belonged to the same litter, the sow having been fed on a diet deficient in fat-soluble vitamins during gestation and the rearing period.

The experiment started on August 10th, 1923. The following is the protocol of the animals:

Pig 133. This animal gained at the rate of 0·6 lb. per day. The first sign of lameness was observed on October 9th and gradually increased; the animal was slaughtered on October 26th.

Pig 136. This animal gained at the rate of 0·4 lb. per day. It was down on October 2nd, and was unable to stand up. It was killed on October 11th. At the post-mortem scorbutic haemorrhages were observed in the left thigh muscle.

Pig 138. This animal gained at the rate of 0·6 lb. per day. It showed first signs of lameness on October 9th which gradually became worse. It was slaughtered on November 8th. At the time of slaughtering the animal manifested pseudo-paralysis of the hind limbs similar to that observed in scorbutic monkeys. At the post-mortem examination numerous large haemorrhages were observed. They were specially large in the left thigh muscle and in the knee joint. Haemorrhages were also observed in the stomach wall and liver.

Pig. 134. This animal gained 0·6 lb. per day. On October 19th it was very weak on its hind legs and was killed on October 26th.

Pig 140. This animal gained at the rate of 0·7 lb. per day. Lameness was first observed on October 12th, which became gradually worse. It was killed on November 30th. No histological examination was made in this case.

Pig 137. This animal did not thrive owing to some intercurrent disease and was, therefore, eliminated from the experiment.

Pigs 132 and 135, although the worst animals of the litter at the beginning of the experiment, grew very well and by November 22nd showed no signs of lameness when the experiment was discontinued. At that time they were in excellent condition.

REPORT ON THE HISTOLOGY OF THE RIB-JUNCTIONS OF THOSE ANIMALS WHICH CAME TO AUTOPSY.

By V. Korenchevsky.

The histological investigation was performed in the usual way, the rib-junction being previously partially decalcified in Müller's fluid.

Pig 124. The line of the zone of provisional calcification is uneven and lime deposition deficient. Protuberances of proliferating cartilage penetrate into the spongiosa. In many places in the centre of the trabeculae of the primary spongiosa, islands of cartilage cells are present. The trabeculae are curved and interlaced, and consist to a considerable extent of osteoid tissue. In the secondary spongiosa osteoid tissue surrounds only some of the trabeculae, and in a diminished degree. Under the periosteum rachitic osteoid proliferations are to be seen.

Diagnosis. Rickets.

Pig. 126. The picture of the costochondral junction of the ribs of this animal is the same as that of Pig 124, with the exception that the amount of osteoid tissue is rather less.

Diagnosis. Rickets.

Pig 128. The histological examination of the ribs shows a convex and uneven line at the zone of provisional calcification with slightly deficient lime deposition, and protuberances of proliferating cartilage into the bone marrow. The primary spongiosa consists of interlaced thin trabeculae, the centre of which contains islands of calcified cartilage cells surrounded by an increased amount of osteoid tissue. The secondary spongiosa does not contain an abnormal amount of osteoid tissue, but the cortical bone does.

Diagnosis. Slight rickets.

Pig 130. Slight osteoporosis only.

Pig 121. Almost normal structure of bone.

Pig 123. Slight osteoporosis only. A photomicrograph of the costochondral junction of this pig is shown in Fig. 10.

Pig 125. The line of provisional calcification is convex, slightly uneven, and in some places calcification is deficient. The trabeculae are thin, but well calcified and do not show an abnormal amount of osteoid tissue.

Diagnosis. Osteoporosis.

Pig 127. The histological examination of the ribs shows the line of zone of provisional calcification to be convex and uneven in some places. Lime deposition is deficient. Here and there, protuberances of the proliferating cartilage penetrate into the bone marrow and the reverse. The primary spongiosa consists of curved interlaced trabeculae, in the centre of which are islands of calcified cartilage cells surrounded by abnormally increased osteoid tissue. The cortical bone and the secondary spongiosa in a lesser degree contain a small increased amount of osteoid tissue.

Diagnosis. Rickets.

A photomicrograph of a section of the costochondral junction from this pig is shown in Fig. 11.

Pig 133. The histological examination of the ribs reveals a picture similar to that of Pig 124.

Diagnosis. Rickets.

Pig 136. The picture of the costochondral junction of the ribs of this animal is similar to that of Pig 127.

Diagnosis. Rickets.

Pig 138. The line of the zone of provisional calcification is uneven, but there is no unusual proliferation of cartilage cells. The primary spongiosa is irregular and some of the trabeculae are surrounded by an increased layer of osteoid tissue. In the deeper spongiosa abnormal osteoid tissue is not seen. Under the periosteum the trabeculae consist of rough calcified bone tissue in the centre which is surrounded by osteoid tissue.

Diagnosis. Mild rickets.

Pig 134. The histological examination of the ribs revealed the line of zone of provisional calcification to be convex and slightly uneven; lime deposition is deficient. The proliferating cartilage is not increased. The primary spongiosa is scanty, its trabeculae irregular and interlaced. Osteoid tissue is slightly increased in the primary spongiosa. In the trabeculae islands of cartilage cells are present. The secondary spongiosa is osteoporotic.

Diagnosis. Very slight rickets with osteoporosis.

The above-mentioned changes in the pigs are very similar to those described by Korenchevsky [1922] on rats kept on a diet deficient only in the fat-soluble factor.

In conclusion I desire to thank Mr Dennis for the photomicrographs which accompany this report.

Conclusion.

From the preceding results it is seen that out of ten animals which went "off their feet," the bones of which have been examined histologically by Prof. Korenchevsky, eight, namely Pigs 124, 126, 127, 128, 133, 134, 136 and 138, showed abnormal amounts of osteoid tissue and some changes in the proliferating cartilage, and in his opinion had been suffering from rickets. Only two failed to show significant amounts of osteoid tissue. The histological picture in these cases was that of osteoporosis. That the rachitic condition was brought about by the consumption of a deficient diet is shown by the fact that on rectifying the ration by the addition of cod-liver oil and fresh full-cream milk (Pigs 122, 129 and 130), the animals were restored to normal health. The constitution of the diet leaves little doubt that it was the deficiency of the antirachitic vitamin which was responsible for the production of the disease, as the diet was complete in all essentials, with the exception of this factor. The striking feature in this investigation is that pigs which developed rickets grew well in the early part of the experiment. In our previous experiments [Golding, Zilva, Drummond and Coward, 1922], the fat-soluble vitamins were more rigorously excluded from the diet with the result that growth was soon greatly impeded, and although a faulty deposition of calcium was demonstrated and a condition of osteoporosis resulted, rickets did not develop. In the present experiments sufficient fat-soluble vitamin A was introduced through the medium of the dried separated milk to induce growth. As has been shown [Zilva, Golding, Drummond and Coward, 1921], very little of the growth-promoting vitamin is necessary for pigs, and the amount supplied was sufficient to produce good growth. When the gain in weight of these animals is compared with that of our previous sets as tabulated below it is seen that not only did these pigs grow much better than the animals on the diets more restricted in the fat-soluble factors (– Ca and – Vitamin A), but that they throve almost as well as the control animals (+ Ca + Vitamin A). We are, therefore, forced to conclude that the presence of a small amount of

the fat-soluble vitamins or vitamin, sufficient to induce growth but not the deposition of calcium commensurate with this rate of development, is a favourable condition for the production of rickets in pigs. This view has been urged by Mellanby in his pioneer work on experimental rickets, by Korenchevsky [1922], and by many other workers since. The presence of large quantities of dried separated milk in a diet fulfils this condition admirably. This is one more instance to add to those brought forward by Mellanby [1921], Korenchevsky [1921, 1922] and Goldblatt [1923] in which rickets is produced by fat-soluble vitamin deficiency alone.

Diet	Weight in lbs. on June 3, 1921 (53 days old)	Gain in lbs. in 54 days
− Vit. A − Ca	29·5	20·8
	30·0	21·0
− Vit. A + Ca	22·5	23·5
	30·5	25·5
+ Vit. A − Ca	28·5	34·6
	26·5	28·0
+ Vit. A + Ca	21·0	30·0
	34·6	40·0

No. of pig	Weight in lbs. on April 21, 1923 (62 days old)	Gain in lbs. in 56 days
124	33·0	38·0
126	41·0	37·5
128	30·5	51·0
127	39·0	44·5
133	40·0	34·0
136	44·5	22·7
138	38·5	32·5
134	39·0	34·5

The results are of interest from the point of view of the practical agriculturist, since pigs are sometimes brought up on rations with a content of the fat-soluble vitamins similar to that of our rachitic diet, and it is therefore not surprising that when apparently thriving they suddenly go "off their feet."

On the assumption that these observations hold true for human beings, the danger of using dried separated milk "fortified" with inactive fats for infant feeding becomes evident.

In this connection we wish to stress the point that the production of rickets in our animals can in no way be associated with a deficiency of mineral constituents in the diet especially of calcium and phosphorus. As will be seen above, these elements were consumed in abundance in the form of inorganic compounds. The ratio of Ca/P ingested was 1/0·6 which, according to the investigations of McCollum, Simmonds, Shipley, and Park [1922], Goldblatt [1924] and Boas and Chick [1924], is not favourable to the production of rickets. We cannot, therefore, support the view of Orr and his collaborators that rickets in pigs is necessarily due to a deficiency of mineral constituents in the diet.

It is of interest to note that two pigs on the diet free from the anti-scorbutic factor developed scurvy. This was fully confirmed by the post-mortem examination of the animals. Plimmer [1920] recorded a case of four

young pigs fed on a diet of meal, sharps, and boiled turnips which developed symptoms conjectured by him to be those of scurvy. On giving raw swedes to these animals the symptoms improved. No post-mortem evidence was, however, produced to show that the animals were really suffering from scurvy. We have made several attempts to induce this disease in pigs by dietetic means and, with the exception of the above mentioned cases, have failed.

In conclusion we wish to express our best thanks to Professor V. Korenchevsky for his valuable help in the preparation and the interpretation of the histological specimens of the ribs.

The greater part of the expenses of this investigation was defrayed from a grant made by the Medical Research Council, to whom our thanks are due.

REFERENCES.

Boas and Chick (1924). *Biochem. J.* **18**, 433.
Goldblatt (1923). *Biochem. J.* **17**, 295.
—— (1924). *Biochem. J.* **18**, 414.
Golding, Zilva, Drummond and Coward (1922). *Biochem. J.* **16**, 394.
Korenchevsky (1921). *Brit. Med. J.* ii, 547.
—— (1922). *Med. Res. Council, Special Report Series*, No. 72, 85.
McCollum, Simmonds, Shipley and Park (1922). *Johns Hopkins Bull.* **33**, 296.
Mellanby (1921). *Med. Res. Council, Special Report Series*, No. 61.
Zilva, Golding, Drummond and Coward (1921). *Biochem. J.* **15**, 427.
Plimmer (1920). *Biochem. J.* **14**, 570.

Fig. 2.

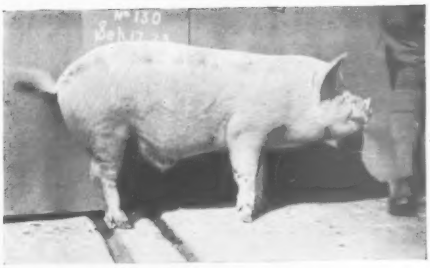

Fig. 3.

Fig. 4.

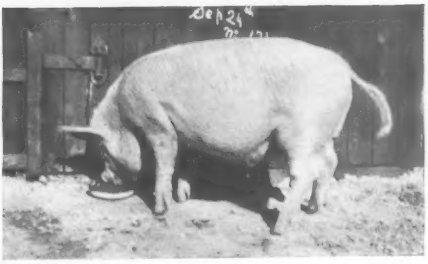

Fig. 5.

Fig. 6.

Fig. 7.

Fig. 8.

Fig. 9

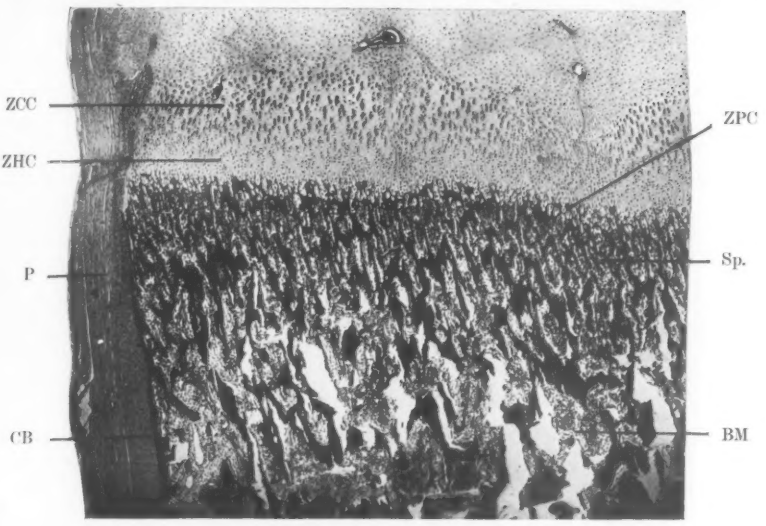

Fig. 10.
Photomicrograph of section of costochondral junction of rib of pig 123 partially cured by sunlight.

Fig. 11.
Photomicrograph of section of costochondral junction of rib of pig 127 showing rickets.

Sp. = Spongiosa. BM = Bone marrow.
ZCC = Zone of columns of proliferous cartilage cells.
ZHC = Zone of hypertrophic cartilage cells.
ZPC = Zone of provisional calcification of proliferous cartilage.
CB = Calcified bone. P = Periosteum.
Ost. = Osteoid tissue. L = Lime deposition.

CXVI. THE STABILITY OF THE VITAMIN A OF COD-LIVER OIL TOWARDS THE HARDENING PROCESS.

By SYLVESTER SOLOMON ZILVA.

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(*Received July 21st, 1924.*)

In another communication reference was made [Goldblatt and Zilva, 1923] to the fact that cod-liver oil retains its high potency as regards growth-promoting and antirachitic properties when hardened. In the case of the growth-promoting property it was demonstrated that an oil retained almost the entire vitamin A content after hardening. Similar observations have since been made by Dubin and Funk [1923].

This note records the results of a series of experiments instituted with the object of studying the stability of vitamin A under different conditions of hardening in order to ascertain the best way of conducting the process with a view of testing such oils clinically. The results obtained show that the activity is hardly affected when cod-liver oil is hardened to certain degrees of hardness even when it is submitted to fairly high temperatures for an appreciable time, providing certain conditions are observed.

The hardening was carried out by Messrs Ant. Jurgens and the author wishes to express his best thanks to this firm for the trouble they have taken and for the readiness with which they met his requests.

In all the experiments great care was taken to exclude all traces of oxygen during the process of hardening. Previous to the treatment air was removed from the oil by exhausting it under an air pump. Care was also taken to use hydrogen free from oxygen.

Samples of hardened cod-liver oil with melting points of 32° and 45° were tested before and after deodorisation. The deodorising was carried out in a glass apparatus with steam at a temperature of 125° at a pressure of about 25 mm. Partially hardened oil was also examined. Such oils although still liquid have lost the unpleasant fishy taste and smell almost to the same extent as the fully hardened oils. All the samples were palatable.

The original oil, which came from Aberdeen, was amber coloured, having an iodine value of 161, a free fatty acid content (calculated as oleic acid) of 2·3 %, and a saponification value of 191, and promoted growth in rats in daily doses of 5 mg. The iodine value of the partially hardened oil fell in one case when it was treated for 43 minutes at 150° to 119. In another case when the treatment was continued for 140 minutes at 150° the iodine value fell to 100·9.

The samples of oil melting at 32° gave an iodine value of 83·3 whilst that of the sample melting at 45° was 54·2.

The hardened oils melting at 32° and 45° were a mixture of oils hardened under various conditions, the details of which are given in the following table:

Sample	Temperature of hardening	Duration of hardening	Decrease in refraction (65°)	Melting point
M.P. 32°	150°	1 hr. 23 min.	0·0090	30°
	150	2	0·0086	33
	150	1 23	0·0090	33
	175	50	0·0094	31
M.P. 45°	150	3 5	0·0115	43·5
	175	2 10	0·0117	45
	175	1 18	0·0117	45
	175	2 10	0·0117	44·5

No appreciable loss in the growth-promoting activity could be recorded in the partially hardened oils and in the oil M.P. 32° whether deodorised or otherwise. The hardened oil M.P. 45° was found less potent than the original oil from which it was prepared. The importance of these facts from a practical point of view needs no comment.

The author wishes to express his thanks to the Medical Research Council for a whole-time grant. Thanks are also due to Messrs Isaac Spencer & Co. Ltd. of Aberdeen for kindly supplying the cod-liver oil used in this investigation.

REFERENCES.

Dubin and Funk (1923). *Proc. Exp. Biol. Med.* **21**, 139.
Goldblatt and Zilva (1923). *Lancet*, ii, 647.

CXVII. THE DISSOCIATION CONSTANTS OF ARGININE.

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(*Received July 21st, 1924.*)

Considering the interest, chemical as well as physiological, attaching to the complex amino acid arginine (α-amino-δ-guanido-valeric acid) it is surprising how incomplete is our information concerning its dissociation constants. The single attempt hitherto made to ascertain their magnitude is that of Kanitz [1906]; but of one only—the second basic—does Kanitz claim to have made an accurate measurement. For the two others—the acidic and the principal basic—he offers, as he himself is careful to state, merely the roughest estimates. The gap thus left in our knowledge we have endeavoured to fill by carrying out an electrometric titration of arginine, the data of which, subjected to appropriate methods of calculation, have yielded satisfactory approximations to the desired constants.

Since it would be extremely difficult to prepare a solution of arginine free from carbonate, we chose as the starting point of our titrations not the base itself, but a salt—the monohydrochloride—which it is easy to obtain in a state of practically absolute purity. The preparation used contained 6·67 % of amino and 26·45 % of total nitrogen (theory, 6·65 and 26·61 respectively); the ratio of amino to total nitrogen was therefore 1:3·97 (theory, 1:4); and the specific rotatory power, $[\alpha]_D^{20°}$, in the presence of seven moles of HCl was + 21·95° [Hunter and Morrell, 1922].

Of this salt we prepared two solutions of nearly identical concentrations. The first (solution *A*) contained 0·8810, the second (solution *B*) 0·8860 g. in 50 cc.; their molar concentrations were therefore 0·0836 and 0·0841 respectively. Of each of these solutions 30 cc. were transferred to a suitable titrating bottle. Each bottle bore a closely-fitting stopper pierced by four holes. The first admitted the tip of a burette, the second the stem of a Moloney [1921] hydrogen electrode, the third a glass tube passing to the bottom of the bottle and serving for the introduction of hydrogen, and the fourth a short tube permitting the exit of the gas. The bottles were immersed in a water-bath of which the temperature, during the titration, was held at 20 ± 0·25°.

For the determination of the first basic and the acid dissociation constants the 30 cc. of solution *A* were titrated with a standard solution of barium hydroxide, protected in the usual way from any access of carbon dioxide.

The strength of this solution was 0·2086*N*, so that 12·0 cc. were the equivalent of the arginine hydrochloride taken. Barium hydroxide was preferred to sodium hydroxide because of the ease with which it may be prepared and maintained free from carbonate. Before titration was started, the titration vessel and its contents were freed from CO_2 by a rapid current of hydrogen, and the flow of gas was maintained uninterruptedly, although at a slower rate, until the entire operation was finished. Titration was effected in steps of 0·5 cc., ample time being given, before each potentiometer reading, for thorough mixing and the establishment of temperature equilibrium. No reading was accepted until it had been thrice repeated without significant alteration. The total amount of baryta solution added was 17 cc., about 1·5 equivalents; this, it will be noted, involved an increase of the volume in the titration bottle by not much more than 50 %.

The procedure described ensures the absence of carbonate, but it involves of course the production of barium chloride. It is open therefore to the objection that the p_H of a buffer mixture, and consequently the apparent dissociation constant of a buffer acid or base, are not unaffected by the presence of a neutral salt. Michaelis and Krüger [1921] found, for example, that a mixture of sodium acetate and acetic acid, each in 0·02*N* concentration, had its p_H reduced from 4·665 to 4·382 by the addition of 0·5*N* barium chloride. But in our titration the concentration of barium chloride at its maximum was no greater than that of the total arginine. It seems, therefore, rather unlikely that it would exert any considerable influence upon the result. Certainly the series of calculated values for $p_{K'_{b_1}}$, exhibited below in Table I, gives no indication of any progressive trend in one direction; yet that is the only kind of effect that the increasing concentration of barium chloride could produce.

The results of the baryta titration, as far as the addition of one equivalent of base, will be found in Table I; while Table II exhibits the effect of carrying the titration further.

The second basic dissociation constant was determined by titrating solution *B* with 0·1815*N* hydrochloric acid. Of this acid 13·9 cc. were equivalent to the 30 cc. of arginine hydrochloride. The titration was carried out in the same way as before, except that the precautions taken to avoid access of CO_2, being here unnecessary, were omitted. The results are shown in Table III.

In all three tables the acid or base in combination with arginine at each step of the titration has been represented in terms of equivalents. This is done chiefly in order to make easy the graphical combination of the separate experiments in the single titration curve of Fig. 1. Since the two solutions, *A* and *B*, were of practically the same concentration, there can be no objection to representing the two experiments thus as one continuous titration. In the figure the circular dots represent experimental points; while the curve as drawn is a theoretical one deduced, from the constants adopted, by the use between p_H 4 and 10 of the well-known Henderson-Hasselbalch equation, and outside that range of the formulae of Van Slyke (see below).

Table I. *Titration of First Basic Group.*

30 cc. of 0·0836*M* arginine hydrochloride titrated with 0·2086*N* barium hydroxide

cc. of barium hydroxide	Equivalents of arginine liberated	Equivalents of HCl left in combination with arginine	$\log \frac{[\text{arginine HCl}]^*}{[\text{arginine}]}$	p_H observed	p_{OH}†	$p_{K'_{b1}}$
0·0	0·000	1·000	—	5·04	9·03	—
0·5	0·0417	0·958	1·36	7·73	6·34	4·98
1·0	0·0833	0·916	1·04	8·08	5·99	4·95
1·5	0·125	0·875	0·85	8·25	5·82	4·97
2·0	0·167	0·833	0·70	8·42	5·65	4·95
2·5	0·208	0·792	0·58	8·52	5·55	4·97
3·0	0·250	0·750	0·48	8·61	5·46	4·98
3·5	0·292	0·708	0·39	8·70	5·37	4·98
4·0	0·333	0·667	0·30	8·81	5·26	4·96
4·5	0·375	0·625	0·22	8·88	5·19	4·97
5·0	0·416	0·583	0·15	8·94	5·13	4·98
5·5	0·458	0·542	0·07	9·03	5·04	4·97
6·0	0·500	0·500	0·00	9·11	4·96	4·96
6·5	0·542	0·458	−0·07	9·20	4·87	4·94
7·0	0·583	0·416	−0·15	9·26	4·81	4·96
7·5	0·625	0·375	−0·22	9·35	4·72	4·94
8·0	0·667	0·333	−0·30	9·42	4·65	4·95
8·5	0·708	0·292	−0·39	9·47	4·60	4·99
9·0	0·750	0·250	−0·48	9·54	4·53	5·01
9·55	0·796	0·204	−0·59	9·67	4·40	4·99
10·0	0·833	0·167	−0·70	9·79	4·28	4·98
10·5	0·875	0·125	−0·85	9·87	4·20	(5·05)
11·0	0·916	0·0833	−1·04	10·04	4·03	(5·07)
11·5	0·958	0·0417	−1·36	10·31	3·76	(5·12)
12·0	1·000	0·000	—	10·67	3·30	—

* The ratio of salt to base is obtained by dividing the figure in column 3 by that in column 2. It may also be calculated as $\frac{12-y}{y}$ where y is the figure in column 1.

† Since the temperature was 20° p_{K_w} is 14·07 [Michaelis, 1922, p. 23] and p_{OH} is $14{\cdot}07 - p_H$.

First basic dissociation constant. The p_H observations recorded in Table I come within the range of applicability of the well-known formula of Henderson [1909] which, as modified by Hasselbalch [1917] and adapted to the case of a base, takes the form

$$p_{K'_b} = p_{OH} - \log \frac{[\text{salt}]}{[\text{base}]}.$$

In this formula K'_b is a constant related to the true dissociation constant, K_b, by the equation $K_b = \gamma K'_b$, in which γ stands for the degree of ionisation of the salt. As γ is seldom far removed from unity, K'_b may for most purposes be taken as a close enough approximation to K_b. Applied to the experimental data, the formula yields, as set forth in the table, consistent results at all points of the titration except those near the end. The divergence of the last three values must apparently be attributed to experimental error, for, as an inspection of Fig. 1 will demonstrate, it cannot be accounted for by the slight overlapping of the basic by the acid titration curve. If these values, bracketed in the table, are rejected, there remain twenty very satisfactory determinations of $p_{K'_{b1}}$ showing an extreme variation of 0·07 and yielding an average of 4·97. This would give to K'_{b1} the magnitude $1{\cdot}07 \times 10^{-5}$.

The true dissociation constant, K_{b1}, may be deduced from the hydrogen ion concentration of the untreated arginine hydrochloride solution (first line of Table I)by the approximative equation [Michaelis, 1922, p. 75].

$$K_b = \frac{K_w \times C}{[H^\cdot]^2},$$

where C is the molecular concentration of the salt, and $\frac{[H^\cdot]}{C}$ represents accordingly the degree to which it is hydrolytically dissociated. K_w at 20° is $0{\cdot}86 \times 10^{-14}$, $[H^\cdot]$ as determined is $0{\cdot}91 \times 10^{-5}$, and C is 0·0836. K_{b1} therefore

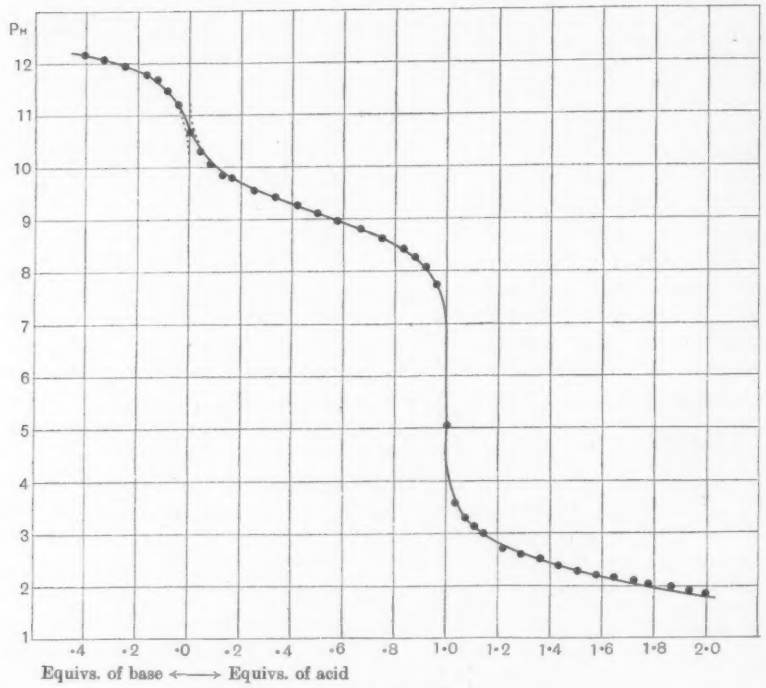

Fig. 1 Titration curve of arginine.

is $0{\cdot}87 \times 10^{-5}$, and arginine hydrochloride in the concentration employed is hydrolysed to the extent of 0·011 %. On this basis γ, the degree of ionisation of the salt, is evidently $\frac{0{\cdot}87}{1{\cdot}07}$, i.e. 0·81 (or 81 %). While these values have all, in relation to K'_{b1}, an entirely reasonable magnitude, it must be remembered that the p_H determination upon which they depend is peculiarly liable to experimental error; for, as the titration curve makes evident, the buffer value of the pure hydrochloride is practically nil.

Table II. *Titration of Acid Group.*

Continuation of the titration recorded in Table I

Barium hydroxide 0·2086*N*

Total cc.	In excess of 12 cc., i.e. of one equivalent: cc.*	equivalents*	*V* cc.	*C*	*B*	p_H observed	p_{OH}	[OH′]	*B′*	*C* − *B′*	$\log \frac{B'}{C-B'}$	$p_{K'_a}$
12·0	0·0	0·000	42·0	·0597	·0000	10·67	3·40	·0004	—	—	—	—
12·5	0·5	0·0417	42·5	·0590	·00245	11·18	2·89	·00129	·00116	·0578	−1·70	12·88
13·0	1·0	0·0833	43·0	·0583	·00485	11·46	2·61	·00245	·0024	·0559	−1·37	12·83
13·5	1·5	0·125	43·5	·0577	·0072	11·67	2·40	·0040	·0032	·0545	−1·23	12·90
14·0	2·0	0·167	44·0	·0570	·0095	11·78	2·29	·0051	·0044	·0526	−1·08	12·86
14·6	2·6	0·217	44·6	·0562	·0122	11·87	2·20	·0063	·0059	·0503	−0·93	12·80
15·0	3·0	0·250	45·0	·0557	·0139	11·95	2·12	·0076	·0063	·0494	−0·89	12·84
15·5	3·5	0·292	45·5	·0551	·0160	12·02	2·05	·0089	·0071	·0480	−0·83	12·85
16·0	4·0	0·333	46·0	·0545	·0181	12·07	2·00	·0100	·0081	·0464	−0·76	12·83
17·0	5·0	0·416	47·0	·0534	·0222	12·16	1·91	·0123	·0099	·0435	−0·64	12·80

* This gives, of course, the quantity of barium hydroxide free to combine with arginine.

Acid dissociation constant. When, as in Table II, the titration passes the stage of free arginine and barium argininate begins to appear, we reach a region of [OH′] where the simple formula employed in the last section no longer furnishes a satisfactory approximation. We have used here therefore an equation developed by Van Slyke [1922] in which the buffer effect of a high concentration of H˙ or OH′ is expressly allowed for. This equation, as applied to the case now in question—that of a very weak acid—takes the form

$$K'_a = \frac{[H^\cdot](B - [OH'])}{C - (B - [OH'])}$$

where C is the total molecular concentration of the arginine in all forms present, and B is the concentration, in terms of normality, of the added base. For purposes of calculation we have found it convenient to represent $B - [OH']$ by the symbol B' and to put the whole equation in the logarithmic form

$$p_{K'_a} = p_H - \log \frac{B'}{C - B'}.$$

The steps of the calculation are set forth in the table, in which V signifies the total volume of liquid in the titration bottle at each step, C is accordingly $\frac{0{\cdot}0836 \times 30}{V}$ and B is $\frac{0{\cdot}2086}{V}$ multiplied by the figure in column 2. The nine values found for $p_{K'_a}$ show, it will be seen, a satisfactory agreement. Their average is 12·84, indicating an acid dissociation constant of approximately $1{\cdot}4 \times 10^{-13}$.

Second basic dissociation constant. This may be obtained from the titration data of Table III by means of the Van Slyke equation for a very weak base

$$K'_{b2} = \frac{[OH'](A - [H^\cdot])}{C - (A - [H^\cdot])}$$

in which C has the same meaning as before and A stands for the concentration of added acid. We have preferred to use this formula also in the logarithmic shape

$$p_{K'_{b_2}} = p_{OH} - \log \frac{A'}{C - A'}$$

where A' signifies $A - [H^+]$. C is calculated in the table as $\frac{0{\cdot}0841 \times 30}{V}$ and A as $\frac{0{\cdot}1815}{V}$ multiplied by the figure in the first column. The formula yields, it will be seen, values of $p_{K'_{b_2}}$ which show a rather greater variability than those obtained for the other constants. The later values in particular show a decided downward tendency. For this reason we have rejected the last three, and taken as a probable approximation the average of the remaining thirteen. This average is 11·90, corresponding to a K'_{b_2} of $1{\cdot}3 \times 10^{-12}$.

Table III. *Titration of Second Basic Group.*

30 cc. of 0·0841*M* arginine hydrochloride titrated with 0·1815*N* HCl.

HCl, 0·1815*N*											
cc. added	Total equivalents present*	V cc.	C	A	p_H observed	$[H^+]$	p_{OH}	A'	$C - A'$	$\log \frac{A'}{C - A'}$	$p_{K'b_2}$
0·5	1·036	30·5	·0827	·00297	3·57	·00027	10·50	·0027	·0800	−1·47	11·97
1·0	1·072	31	·0814	·00584	3·28	·00053	10·79	·0053	·0761	−1·15	11·94
1·5	1·108	31·5	·0801	·00864	3·14	·00073	10·93	·0079	·0722	−0·96	11·89
2·0	1·144	32	·0789	·0113	2·98	·0011	11·09	·0102	·0687	−0·83	11·92
3·0	1·216	33	·0765	·0165	2·72	·0019	11·35	·0146	·0619	−0·63	11·98
4·0	1·288	34	·0742	·0213	2·60	·0025	11·47	·0188	·0554	−0·47	11·94
5·0	1·360	35	·0721	·0259	2·52	·0030	11·55	·0229	·0492	−0·33	11·88
6·0	1·432	36	·0701	·0302	2·39	·0041	11·68	·0261	·0440	−0·23	11·91
7·0	1·504	37	·0682	·0343	2·30	·0050	11·77	·0293	·0389	−0·12	11·89
8·0	1·575	38	·0664	·0382	2·20	·0063	11·87	·0319	·0345	−0·05	11·92
9·0	1·647	39	·0647	·0419	2·16	·0069	11·91	·0350	·0297	+0·07	11·84
10·0	1·719	40	·0631	·0454	2·11	·0078	11·96	·0376	·0255	+0·17	11·79
11·0	1·791	41	·0616	·0487	2·03	·0093	12·04	·0394	·0222	+0·25	11·79
12·0	1·864	42	·0601	·0519	2·00	·0100	12·07	·0419	·0182	+0·36	(11·71)
13·0	1·935	43	·0587	·0548	1·91	·0123	12·16	·0425	·0162	+0·42	(11·74)
14·0	2·006	44	·0574	·0577	1·84	·0145	12·23	·0432	·0142	+0·48	(11·75)

* Including the one already combined in the hydrochloride.

Iso-electric point. The iso-electric point of a mono-acid, mono-basic ampholyte may be calculated by the formula

$$p_I = \frac{p_{K_a} + p_{k_b}}{2},$$

in which p_{k_b} means $p_{K_w} - p_{K_b}$. The second basic dissociation constant of arginine is so far removed from the first that this simple formula may be applied here without sensible error [Levene and Simms, 1923]. The iso-electric point of arginine is therefore approximately $\frac{12{\cdot}84 + 9{\cdot}10}{2} = 10{\cdot}97$ in terms of p_H, or, in terms of $[H^+]$, $1{\cdot}1 \times 10^{-11}$.

Discussion

From measurements (not reported in detail) of the conductivity of arginine mononitrate solutions (prepared by mixing the dinitrate with one equivalent of sodium hydroxide) Kanitz concluded that this salt is not measurably hydrolysed. The conclusion was confirmed by the failure of a $M/10$ solution to cause

appreciable hydrolysis of methyl acetate. Kanitz[1] made the only deduction which such observations could justify—that the principal basic dissociation constant of arginine is "at least 1×10^{-7}." The hydrolysis of the hydrochloride, and presumably therefore of the nitrate, is indeed, as our results have shown, exceedingly slight, and Kanitz's statement with regard to the constant is accordingly literally correct; but the words "at least" must, in the light of our measurements, be now interpreted to mean "one hundred times greater than."

Our value for K'_{b1} ($1{\cdot}07 \times 10^{-5}$) shows arginine to be a base nearly as strong as ammonia, of which the dissociation constant (at 18°) is $1{\cdot}75 \times 10^{-5}$. Among acids acetic acid has a constant ($1{\cdot}82 \times 10^{-5}$) of a similar order of magnitude.

The second basic dissociation constant was derived by Kanitz from measurements of the catalytic action of arginine dinitrate upon the saponification of methyl acetate. The value found was $2{\cdot}2 \times 10^{-12}$ ($p_{K_{b_2}} = 11{\cdot}66$). This was the only one of his determinations in which Kanitz himself expressed particular confidence. It stands in very fair agreement with our own estimate of $1{\cdot}3 \times 10^{-12}$ ($p_{K'_{b_2}} = 11{\cdot}90$). If both estimations had been carried out at the same temperature, the agreement might have been better still; for K_b generally increases rather rapidly with rising temperature [Lundén, 1906], and Kanitz's result was obtained at a temperature of 25°.

The second basic constant of arginine, though far smaller than the first, is still of as high an order of magnitude as the basic constants of many monoamino acids; thus for aspartic acid $K_b \times 10^{12}$ has the value 1·2, for phenylalanine 1·3, for leucine 2·3, for tyrosine 2·6, for glycocoll 2·7.

In an attempt to measure the strength of arginine as an acid Kanitz mixed two moles of arginine dinitrate with three of sodium hydroxide and from the conductivity of the mixture deducted that of two moles of sodium nitrate. The conductivity of sodium-arginine, thus obtained, appeared to be approximately the same as that of sodium hydroxide. Kanitz concluded that K_a is less than $1{\cdot}11 \times 10^{-14}$ and that arginine has in fact no acid properties at all. It is not surprising that the method described led to uncertain results, or that the conclusion reached has not been entirely confirmed by our own observations. The latter lead to a constant, $1{\cdot}4 \times 10^{-13}$, which is not quite so small as Kanitz believed it. It is small enough, nevertheless, to characterise arginine as a very weak acid indeed, much weaker even than phenol, hydrocyanic acid, or asparagine, which have acid dissociation constants of 1·3, 4·7 and 8·8 times 10^{-10} respectively, and comparable only with such excessively feeble acids as sucrose ($K_a = 1{\cdot}14 \times 10^{-13}$), glucose ($3{\cdot}6 \times 10^{-13}$) and fructose ($6{\cdot}6 \times 10^{-13}$). It may be of further interest to note that the strength of the di-acid base arginine as an acid is of an even lower order than the strength of the di-basic acid aspartic acid as a base; for the basic dissociation constant of the latter is $1{\cdot}2 \times 10^{-12}$.

[1] As pointed out by Harris [1924], Kanitz's tabulation of his results contains misprints which completely distort some of his actual conclusions.

The constants for substances other than arginine, mentioned in the preceding paragraphs, have been taken from the compilations of Lundén [1906] and Michaelis [1922].

Summary.

Arginine mono-hydrochloride was titrated with (*a*) barium hydroxide, and (*b*) hydrochloric acid. From the hydrogen-ion concentrations of the resulting mixtures the following approximations to the dissociation constants and isoelectric point of arginine were derived:

$$K'_{b1} = 1{\cdot}07 \times 10^{-5};\ p_{K'_{b_1}} = 4{\cdot}97$$
$$K'_{b2} = 1{\cdot}3 \times 10^{-12};\ p_{K'_{b_2}} = 11{\cdot}90$$
$$K'_{a} = 1{\cdot}4 \times 10^{-13};\ p_{K'_{a}} = 12{\cdot}84$$
$$I = 1{\cdot}1 \times 10^{-11};\ p_{I} = 10{\cdot}97$$

References.

Harris (1924). *Proc. Roy. Soc. London*, B, **95**, 440.
Hasselbalch (1917). *Biochem. Z.* **78**, 112.
Henderson (1909). *Ergeb. Physiol.* **8**, 254.
Hunter and Morrell (1922). *Trans. Roy. Soc. Canada*, **16**, v, 75.
Kanitz (1906). *Z. physiol. Chem.* **47**, 476.
Levene and Simms (1923). *J. Biol. Chem.* **55**, 801.
Lundén (1906). *Z. physikal. Chem.* **54**, 532.
Michaelis (1922). Die Wasserstoffionenkonzentration, Teil 1, Berlin.
Michaelis and Krüger (1921). *Biochem. Z.* **119**, 307.
Moloney (1921). *J. Physical Chem.* **25**, 758.
Van Slyke (1922). *J. Biol. Chem.* **52**, 525

CXVIII. STUDIES ON INOSITOL.

II. THE SYNTHESIS OF INOSITOL IN THE ANIMAL BODY.

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Inositol, the most important member of the cyclose group, once thought to be a carbohydrate, and now known to have the formula of hexahydroxycyclohexane, appears to be a constant constituent of all animal and vegetable cells. But in spite of its exceedingly wide distribution, and the considerable amount of work which has been carried out upon it, we are still ignorant of its functions and metabolic relationships. In approaching such a subject there would seem to be certain steps which must inevitably be taken before all else, and the first of these is the elaboration of a satisfactory method of estimation, while the second is to find out whether or not the animal body has the power of synthesising the substance. Such information is absolutely necessary for further experiment, and little can be done until it is gained. In the first of these papers [1923, 1] there was described a method of estimating inositol, which has an accuracy of 90 % and which is capable of being employed upon as little as 12 g. of tissue. The next step was to try to find out whether the animal body has the power of synthesising the inositol ring.

Previous workers, no doubt owing to the absence of a method of estimation, had made no attempts to attack the problem directly, but indications of various kinds were not wanting. Vohl [1858] had reported that from the urine of a case of diabetes insipidus he had succeeded in isolating from 18 to 20 g. of inositol per diem. In view of the imperfect methods of isolation which were in use at that date, his data might seem doubtful, had not Hopkins [1923] found a very similar case, excreting about 15 g. every day. Such quantities of inositol could certainly not be coming in in the food, especially as inositol is readily broken down by *B. coli* in the intestinal tract. No other conclusion is possible except that synthesis must have taken place. Offergeld [1906] could find no inositol in the blood of a pregnant woman, and as the tissues of young children are known to contain it, concluded that the foetus must be synthesising its own. Little importance can be attached to this observation as Offergeld only used a litre of blood, and blood only contains inositol in very low concentrations. The present writer was able to isolate 1·1 mg. % from the blood of the goat. Klein [1909] was unable to isolate any inositol from the

unincubated hen's egg, but found some present in the hatched chick and therefore concluded that a synthesis had taken place. These observations will be returned to later. Starkenstein [1909], in the course of a general investigation into the metabolism of inositol, starved rabbits for eleven days, and at the end found no diminution in the inositol-content of their muscles. His conclusion was that inositol was not broken down in the body, but the experiment is not conclusive, for it takes no account of the considerable amounts of combined inositol which exist in the body, and can as easily be explained by saying that what he was measuring was a condition of maintained equilibrium. This experiment is also no evidence that synthesis can take place for the same reasons.

It seemed clear that little help could be expected from the older literature. Apart from all other considerations, no worker had ever taken care to control the diet, and rats, animals which live well on a "pure" diet composed of pure carbohydrate, protein, and fat, had never been used as experimental material. The problem obviously needed a new line of approach.

At this point another series of papers seemed important in this connection. Diabetes insipidus, with its related condition, inosituria, is a pathological state which has been attracting the attention of clinicians for over a century. It was only distinguished in the early years of the nineteenth century from diabetes mellitus by the fact that there was no sugar in the urine, hence the name "zuckerlöse Harnruhr." But Cloetta [1856], Vohl [1858], and Neukomm [1860], all observed an inosituria accompanying cases of diabetes insipidus, to a greater or less degree. For a time, the fact was expected to throw much light on the etiology of the disease, but it did not do so, and for the following fifty years many workers were trying to associate it, either with diabetes insipidus, diabetes mellitus, or simple polyuria. It was soon found that by no means all cases of diabetes insipidus were also inosituric [Ebstein, 1873; Bürger, 1873], while inosituria was found by Neukomm [1860], Gallois [1864], Mosler [1868], Przibram [1871] and Leva [1891] to be sometimes associated with diabetes mellitus. Külz, however [1875, 1876], made the observation that inositol was excreted to a marked extent in the polyuria caused by an excessive drinking of beer by two German students. Since wine and beer contain inositol this experiment was not conclusive, but he also discovered that the injection of physiological salt solution into rabbits caused an inosituria, as also did the ingestion of large amounts of water in man. From these experiments, there resulted the idea that inosituria was specially to be associated with polyuria, and only appeared in the two types of diabetes when these were complicated by an excessive water-excretion. This view was put forward by Mayer [1907] and Starkenstein [1909] and it was greatly stressed by the latter as it fitted in with his general theory that the inositol of the tissues entered the body in the form of phytin, and possessed no physiological function. In polyuria, it was just being washed out. Meillère [1906] obtained an inosituria by puncture of the fourth ventricle and stated that the inositol reached its

maximum two hours before the glucose, though he gave no figures in his paper.

There can be no doubt at the present time that inosituria is associated with polyuria of whatever origin, and that its connection with the two forms of diabetes is accidental. If, therefore, a number of rats could be made to undergo artificial polyuria, while at the same time living on a pure diet, from which all inositol was excluded, good evidence would be available on the question of synthesis by the animal body. For an inosituria would accompany the polyuria, and, since no inositol was entering their bodies in their food, it would become clear whether or not they could synthesise it. If after some time the excretion of inositol diminished and ceased although the polyuria still continued, we should have a direct proof that the body was incapable of effecting its synthesis. If, on the other hand, the excretion of inositol was kept up indefinitely in spite of the supplies being cut off, then we should have to conclude that it was being synthesised. The description of such an experiment occupies the main part of this paper.

First, however, we shall describe some experiments with pure diet only.

Experiments with rats on a pure diet.

Quite apart from the effects of a prolonged polyuria, the result of cutting off all cyclose from the diet appeared likely to throw some light on the question of synthesis. Accordingly a number of white rats were placed on a "basal diet" and kept for a long period, one or two being from time to time removed and their inositol-content estimated, as described in the previous paper [1923, 1]. The diet was that in general use in the Cambridge Laboratory; it consisted of pure caseinogen, sucrose, and palm-kernel oil as fat, with the addition of a minute quantity of marmite extract and orange juice. The marmite was worked up for inositol, and traces were found in it. Since, however, only three drops were given to each rat every day and free inositol in the food is broken down by intestinal bacteria, this factor would not be expected to influence the results greatly. The figures obtained in this way are given in Table I.

Table I.

		Weights		Inositol found		
Date when rat was killed	Rat number	Original weight	Total tissues	Total mg. per rat	mg. % orig. weight	mg. % total tissues
23 Oct. 1923	TRB	76·0	61·40	8·67	11·41	14·1
31 Oct. 1923	TRE	70·0	46·5	6·34	9·06	13·63
	TRF	96·0	64·0	4·97	5·17	7·76
17 Nov. 1923	TRJ	110·0	70·70	21·15	19·23	9·91
17 Mar. 1924	TRO	247·0	132·0	14·96	6·06	11·33
12 May 1924	TRP	268·0	153·0	16·15	6·03	10·55

The normal limits of variation for the inositol-content of rats are between 7 and 14 mg. % calculated with reference to the original body weight, and the above table shows that over a period of eight months the inositol-content of rats kept on an inositol-free diet varies only within those limits. In all cases

the whole body of the rat was included in the estimation, and all that was rejected was the skin and the intestinal contents. The "original weight," therefore, refers to the weight of the animal before death, and the term "total tissues" refers to the weight of the material before extraction with acetone. Death was in all cases brought about by chloroform. These conditions were those under which all the experiments in this paper were carried out.

These experiments seem to point to the view that the body can synthesise inositol, always assuming, of course, that inositol undergoes normally a breakdown in the body. It is true that, as will appear later, there is a good deal of combined inositol present in the body, but nevertheless the fact that rats on an inositol-free diet show no diminution after eight months, in the amount directly estimable in their bodies, can surely not be without significance.

Experiments on rats with artificially induced polyuria.

Methods.

As a means of producing a continuous polyuria, recourse was had to a very salt diet. Injection of salt solution and other diuretics was impossible in an experiment planned, as this was, to last months; something that could be taken by the mouth was necessary, and the least unphysiological substance that could be thought of was sodium chloride. It was increased gradually and finally kept at a concentration of 17 % in the food. This quantity did not seem to hurt the animals at all, and certainly succeeded in raising and maintaining a vigorous polyuria, as will be seen later.

The bodies of the rats were treated in the manner described in the last paper for the estimation of inositol, and no modifications were found to be necessary for them. The estimation of inositol in the urine was, however, a more difficult matter and one or two important modifications had to be introduced. In the first place, the earlier workers seem to have taken no precautions to keep the urine of their animals sterile, and in view of the rapidity with which bacteria break down the cycloses, this is a distinct error. *B. coli*, according to Graham-Smith [1924], is common in urine, especially that of rats, and inositol is broken down readily by it [Meillère, 1907; Starkenstein, 1909, 1911]. Consequently, it was decided to collect the urine in an antiseptic, and chlorine water was chosen, firstly, because it is very toxic to *B. coli*, and secondly, because it passes off when the urine is evaporated down. Some preliminary experiments were made to determine whether it had real protective power. A culture of *B. coli* was sown into one flask containing 25 cc. of rat's urine, and 0·05 g. inositol added. A similar flask was prepared but with the addition of 5 cc. of chlorine water. From the first 0·038 g. was recovered, and from the second 0·048 g. Accordingly in all subsequent experiments 10 cc. of chlorine water were added to the vessel in which the urine was collected.

The second modification was concerned with the removal of the inorganic

salts of the urine before the precipitation with basic lead acetate. Mayer [1907], Anderson [1916] and Greenwald [1917] all used neutral and basic lead acetate precipitations direct on the urine, but they admitted that their method was only approximate. Inorganic salts are known to hinder the precipitation of cycloses by the basic lead salt [Momose, 1916], and these were removed in Starkenstein's method [1909] by a series of precipitations with barium and silver nitrates, sodium phosphate, etc. In order to avoid this, the urine was evaporated down to about 500 cc. and plaster of Paris added. The mass does not set hard but forms a dry powder which can easily be extracted with dilute acetone as described for tissues in the first paper of this series. This yielded satisfactory results, 0·24 and 0·228 g. being recovered from 0·25 g. added to 200 cc. of urine in two experiments and was greatly superior to Starkenstein's method (0·120, 0·103, 0·164 g. recovered).

The rats were confined in cages large enough to hold six animals comfortably. Each cage was furnished with a wire bottom and four tunnels leading out of it at the sides. Underneath the cages were funnels which collected the urine into thick glass vessels. Two of the tunnels led to food while two led to water. A continual drip of water was arranged, so that the drinking-troughs were always full and the animals were never thirsty. By this means the polyuria was kept up continuously.

Experiments.

Two preliminary experiments were first performed. In order to try to confirm the work of Külz [1876] one of the workers in the laboratory consented to drink a large quantity of water. He ingested between 2.30 and 6.0 p.m. a little over seven litres of water, and excreted between 3.27 and 7.28 p.m. 4210 cc. of urine. From this amount, it was possible to isolate 0·45 g. of inositol. Next, 15 rats were made to excrete a considerable amount of urine by giving them 5 % of salt in their diet, and their urine was worked up for inositol. The results were eminently satisfactory. Not only did a marked diuresis appear, but inositol was also present, as is seen from Table II.

Table II.

	cc. urine per rat per day	Total mg. inositol excreted	mg. inositol per day per rat
Normal (3 rats)	15·5	0·0	0·0
Polyuria (15 rats)			
First day	38·0	10·07	0·72
Second day	66·67	12·95	0·86
Third day	93·33	7·61	0·51
Fourth day	60·66	11·80	0·79

From these results it was obvious that a polyuria could be induced in rats, that it could be accompanied by an inosituria and that the inositol was excreted in easily measurable amount. Nothing remained but to see how long this could continue on a pure diet.

Eighteen large rats, weighing *in toto* a little over seven kilos., were there-

fore confined in four cages of the type described, and placed on a pure diet for three days to accustom them to it. Then sodium chloride was added to their food in gradually increasing quantities, rising to 15 %. Table III and Fig. 1 show the results which were obtained.

Table III.

24-hour period	Total amount of urine cc.	% salt in diet	cc. urine per rat per day	mg. inositol excreted		
				Per day	Per day per rat	% of urine
1	270	5	15·5	5·15	0·286	1·845
2	510	9	28·3	14·21	0·789	2·785
3	615	12	34·2	22·49	1·249	3·655
4	810	15	45·0	9·86	0·547	1·215
5	1040	15	57·7	11·00	0·611	1·058
6	1370	15	76·1	[14·50]	[0·805]	—
7	1840	15	102·2	18·67	1·035	1·013
8	1700	15	94·4	[14·50]	[0·805]	—
9	845	15	46·9	11·17	0·620	1·320
10	2100	15	116·6	[17·00]	[0·944]	—
11	2030	15	112·8	22·93	1·274	1·130
12	2120	15	117·8	30·59	1·699	1·445
13	2280	15	126·7	17·39	0·965	0·761
14	2110	15	117·2	[13·00]	[0·722]	—
15	2240	15	124·5	9·46	0·504	0·405
16	2360	15	131·1	23·66	1·314	1·002
17	1720	15	95·5	50·58	2·809	2·941
18	2270	15	126·1	36·98	2·054	1·630
19	1800	15	100·0	30·93	1·718	1·718
20	2020	15	112·2	22·37	1·243	1·111
21	2430	15	135·0	78·66	4·369	3·237
22	2260	15	125·5	47·90	2·661	2·120
23	2220	15	123·3	39·42	2·190	1·776

Total mg. inositol excreted per rat ... 27·837
Calculated by interpolation ... ... 3·276
31·113

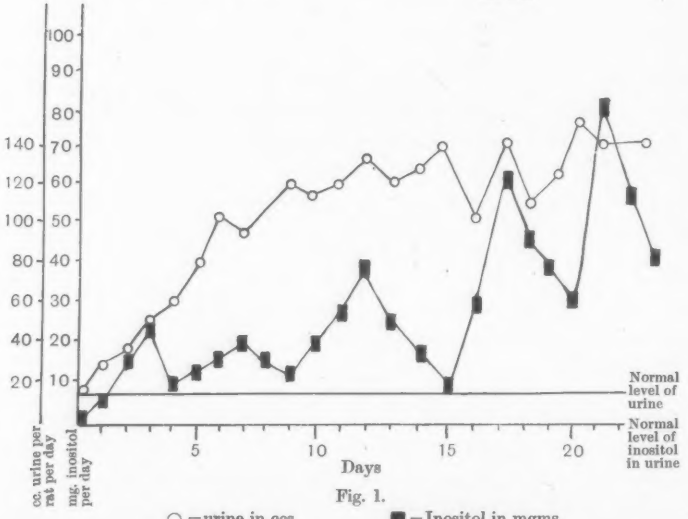

Fig. 1.

○ = urine in ccs. ■ = Inositol in mgms.
The size of the black squares indicates the possible error.

It is clear, therefore, that over a period of 23 days' polyuria, during which the excretion of urine was raised to as much as nine times the normal, inositol was continuously excreted. But the most interesting thing that appears from Fig. 1 is the rhythm with which this was done. In peaks of gradually ascending height the concentration of cyclose in the urine rises until it is about eight times the ordinary excretion. On the twenty-third day the experiment was stopped and most of the animals killed for the estimation of the inositol that they contained. Would the difference in their body-content account for the 31 mg. which they had each excreted, or would a synthesis have to be postulated? The results are given in Table IV.

Table IV.

		Weights		Inositol		
Conditions	Rat number	Total tissues	Original animal	mg. per rat	mg. % of total tissues	mg. % orig. weight
Normal	B	26·5	64·0	5·78	9·03	21·81
,,	E	28·0	70·0	6·63	9·47	23·68
,,	HC	50·0	93·0	6·74	7·24	13·48
,,	WC	38·0	77·0	7·81	10·15	20·56
23 days' polyuria	EP	234·0	384·0	22·06	9·43	5·745
,, ,,	EE	189·0	311·0	18·06	9·428	5·81
,, ,,	N	205·0	250·0	16·86	8·23	6·75

It is obvious that there has been no change in the free inositol estimable directly, before and after the polyuria. It does not even vary greatly within the normal limits of change, but remains almost constant at 9 mg. %. However, there remains still the question of "inositogen." The first worker to suggest the presence in the tissues of a precursor of inositol, capable of being split down to inositol by tissue enzymes was Rosenberger [1908, 1909, 1910]. His first experiments were not very definite, for he compared the amount of inositol present in beef which had been exposed for sale with that present in fresh rabbit muscle, having ascertained for neither the limits of normal variation. However, in later work, he suspended minced rat muscle in salt solution and autolysed it for three days, after which he was able to isolate very much more inositol than he could from the fresh muscle. Unfortunately, he gave no figures, and his method, which involved boiling with concentrated HNO_3 and baryta, received much just criticism [Starkenstein, 1908]. He ventured no distinct view as to what the precursor might be except to suggest that it might be the "tyrophenositol" of Müller [1907] and Danileffski [1880]. Starkenstein [1909] could not succeed in obtaining the differences that Rosenberger had reported, and threw doubt on the existence of an inositogen, although he thought that if it existed it might be phytin or a phytin-like compound, absorbed from the intestine. The presence of a phytase in certain tissues, reported by McCollum and Hart [1908] seemed to favour this belief. The present writer, moreover, was able to isolate in 1923 more inositol from a rabbit which had been dead two days than from one freshly killed. Accordingly, although polyuria continued for 23 days seemed to have no effect upon the

free inositol of the rat, it was possible that it had decreased the store of combined inositol, kept the amount of free substance at a constant level, and rendered the experiment no proof of synthesis. To test this point, autolysis experiments were carried out and are shown in Table V.

Table V.

Rat number	g. of muscle	Duration of autolysis in hours	Inositol mg. isolated	Inositol mg. % of muscle	Conditions
A–B	40	0	9·17	22·92	After 23 days' polyuria
		4	20·01	50·06	
		4	16·15	40·36	
		12	52·48	134·3	
		12	54·05	135·1	
TH–M	31·5	0	2·98	9·48	After 23 days' polyuria
		4	17·99	57·11	
		4	12·03	38·19	
		12	55·03	174·70	
		12	54·04	171·60	
CH–T	30·0	0	11·4	38·0	Normal
		4	13·87	46·25	
		17	104·7	341·00	
		17	71·18	237·30	

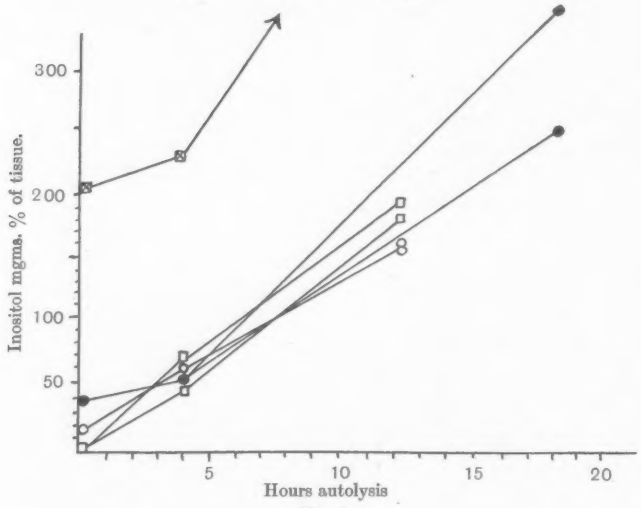

Fig. 2.

● Normal. ⊠ 70 days' polyuria.

□ ○ 23 days' polyuria.

Two rats, which had been under similar conditions, were taken, and enough of their skeletal musculature minced to provide five portions of about 30 g. each. One of these was estimated directly, and four were suspended in 250 cc. of phosphate buffer solution [Cole, 1919] at a p_H of 7·00. To each of these 10 cc. of toluene were added and two of them allowed to autolyse for 4 hours, while two were left for 12 hours, all at 37°.

These figures may be considered strong evidence in favour of the existence of inositol in the combined state capable of being liberated on autolysis. It must, however, be remembered that in a mixture of substances such as autolysis produces, there is always the possibility that unknown bodies may be estimated as inositol. But the figures also showed that the experiment just described was not quite conclusive proof that a synthesis of inositol had been going on to supply the excreted substance. For although the figures for total body-content had remained the same there was a slight diminution in the amount of inositol capable of being liberated by 12 hours' autolysis, and this was still the case even when the longer autolysis in the control was allowed for (see Fig. 2). Moreover, the gradually increasing rhythmic output of inositol shown in Fig. 1 led naturally to the question of how long this would continue. Accordingly, it was decided to repeat the whole experiment, employing just the same conditions, and a very much longer time.

Since this second experiment was planned to cover several months, it was not possible to estimate the inositol in each day's urine; instead, the urine was kept at − 2° for five days, and then the massed amounts estimated in five-day periods. The salt content of the food was held at 17 % in lieu of 15 %, and the rats were allowed a week to get used to their pure unsalted diet, but otherwise there was no change in the conditions. From time to time tests were made on the urine for glucose and albumin; but in no case was glucose found (Benedict) while albumin only appeared about the 80th day (sulphosalicylic acid). Creatine was found to be present [cf. Fowler and Hawk, 1910].

Table VI.

				Urine cc.			Inositol			
No. of days	5-day period	% of salt	No. of rats	Total per period	Average per day	Average per rat per day	Total mg. per period	mg. per day	mg. per rat per day	mg. %
5	1	10	18	4275	855	46·7	9·0	1·8	0·100	0·210
10	2	12	18	8690	1738	96·5	88·5	17·7	0·983	1·019
15	3	15	18	9660	1932	107·3	37·0	7·4	0·411	0·383
20	4	17	18	13050	2600	145·0	1019·7	203·9	11·320	7·841
25	5	17	18	9660	1932	107·5	785·0	157·0	8·722	8·127
30	6	17	18	10240	2048	113·9	—	—	—	6·0?
35	7	17	18	8180	1636	93·1	58·1	11·6	0·644	0·709
40	8	17	18	11070	2214	131·9	92·2	18·4	1·022	0·830
45	9	17	16	10380	2076	129·75	119·1	23·8	1·488	1·147
50	10	17	16	10635	2127	132·9	193·6	38·7	2·418	1·820
55	11	17	16	9794	1959	122·12	158·8	31·7	1·975	1·618
60	12	17	16	9340	1868	116·96	—	—	—	0·80?
65	13	17	16	9790	1958	122·57	34·4	7·0	0·437	0·357
70	14	17	10	7540	1508	150·8	340·3	68·1	6·810	4·516
75	15	17	10	7910	1582	158·2	271·0	54·2	5·420	3·423
80	16	17	10	8250	1650	165·0	228·5	45·7	4·570	2·770
85	17	17	10	8470	1694	169·4	351·0	70·2	7·020	4·143
90	18	17	10	6560	1640	164·0	198·8	39·7	3·970	2·421
95	19	17	10	7520	1540	150·4	73·0	14·6	1·460	0·971
100	20	17	9	8670	1734	192·4	126·7	25·3	2·800	1·459
105	21	17	8	9240	1850	230·9	62·0	12·4	1·550	0·670
110	22	17	9	6760	1352	148·8	50·5	10·1	1·100	0·747
				Total mg. of inositol excreted per rat			...	64·220		
				Calculated by interpolation			...	6·800		
								71·020		

From Table VI and from the graphic representation of the same figures (Fig. 3), there remains no doubt that over a period of 110 days of artificially induced polyuria, inositol is constantly excreted. The total amount actually excreted by one rat during the whole period amounts to 71 mg., and in the absence of any inositol from the diet, the evidence for synthesis is complete. The second experiment is in good correspondence with the first, because in both cases the maximum excretion occurs about the third week. In the first case it was actually in the 21st day, in the second case about the 19th

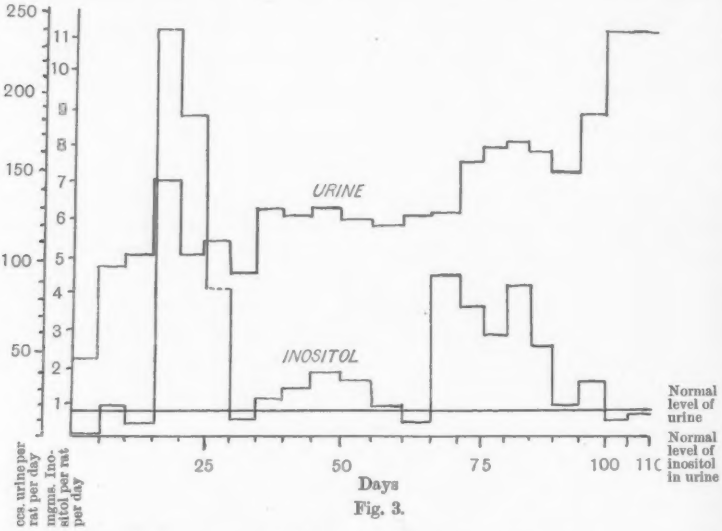

Fig. 3.

day, at any rate, between the 15th and the 20th days. The first experiment ended shortly after, but in the second one, a fall to a constant level takes place, and the excretion continues steadily for some time at a level of from 1·5–2·5 mg. per rat per day. Thereafter, other changes occur. The number of rats is reduced to 10 at the 70th day, to 9 at the 100th day, and lastly to 8. Profound histological changes take place in the kidneys, and then the output of cyclose rises.

In order to make sure that the continuing output of inositol was not due to a mere depletion of the inositol liberated by overnight autolysis, similar autolysis experiments to those described above were carried out on the four rats killed on the 70th day. The results are given in Table VII.

These figures, when compared with those in Table V, show very large increase in the amount of free inositol as well as in that of the inositol liberated by overnight autolysis. On the other hand, the amount of inositol liberated by 4 hours' autolysis is relatively lower than the normal. The exact interpretation of these figures can hardly be attempted till we know more of the

ordinary course of autolysis in muscle as regards the cyclose group, yet at the same time they do show that whatever else has happened, there has been no decrease in the inositol of the body after prolonged inosituria on pure diet. Perhaps an over-compensation has taken place.

Table VII.

Rat number	g. of muscle	Length of autolysis in hours	Inositol: mg. isolated	Inositol: mg. % of muscle	Conditions
TH–PH	23·0	0	46·62	202·7	70 days' polyuria
		4	54·2	235·7	
		20	198·7	864·2	
		20	218·1	948·2	

How had the kidneys behaved under this strain? At the 45th day two rats died. They were very puffed up, their abdominal distention being due not to liquid but to gas in their intestines. This must probably not be considered as having anything to do with the salt diet, for death with abdominal distention is comparatively frequent when rats are fed for some time on a pure diet. Their kidneys were excised and placed in Carnoy's fluid to await histological examination. Then on the 70th day four further rats were killed, and from two of them kidney sections were made.

The sections reveal a progressive degeneration. At 45 days there is some breakdown of the tubule cells, though this is more marked in some areas than others. In other places there is evident a clumping together of nuclei, though on the whole the tubules are of normal shape. But the most remarkable point is the great shrinking of the glomeruli, a phenomenon not due to the method of preparation, for the controls did not show it. Instead of filling up the whole glomerular space, the glomerulus only fills half of it or so.

After 25 more days' polyuria, the degeneration is much more marked, especially as regards nuclei. There is hardly a single nucleus left, and the tubules have largely lost their shape. Masses of pigment are seen in a few places and in most of the glomeruli the nuclei are entirely absent. The whole picture is one of enormous nuclear degeneration, together with general breakdown of cells.

These appearances coincide well with the few data that we have as to the histological appearances of kidneys in human beings who died from diabetes insipidus or other forms of excessive polyuria. The only available information on this point seems to be that given by W. Roberts [1865]. In six post-mortems of diabetes insipidus cases, three kidneys were much diminished in size, and three were very large and flaccid. But histologically, the same appearances were present in each, shrunken glomeruli, degenerating tubules, and in the worst cases, disappearance of the characteristics of kidney tissue. Degeneration, then, seems to accompany excessive and long-continued polyuria, and this fact explains why the constancy in inositol excretion seen in the 6th to the 13th periods is not maintained. Probably after a certain time, the

threshold for most substances is altered by the degeneration, and inositol begins to leak through. The constant level of inosituria can no longer be maintained.

Discussion.

Two lines of evidence point to the conviction that inositol is capable of being synthesised by the animal body. We have seen that there is no change in the inositol content of rats after eight months on an inositol-free diet, and we have seen also that on the same diet, a vigorous inosituria causes no diminution of the body-cyclose. These are supported by a third, the behaviour of inositol in the developing egg. It was shown [Needham, 1923, 2] that the inositol content of the unincubated hen's egg is about 6 mg. %, while at the end of development it has risen to 60 mg. %. Now if all this inositol had existed in the form of a phytin-like compound at the beginning, and even if it had been in the form of a monophosphate, about double the water-soluble phosphorus present at the beginning would have been needed, using the figures of Plimmer and Scott [1909]. Accordingly, unless some compound other than a phosphorus one is postulated as the precursor of inositol, the conclusion is that a synthesis of inositol has taken place. Though this is an indirect consideration, nevertheless it reinforces the other lines of evidence.

Four modes of reaction might have occurred in the polyuria experiments:

1. Excretion ceases after a time. Body-content lowered.
2. „ „ „ „ raised or normal.
3. Excretion continues indefinitely. Body-content lowered.
4. „ „ „ „ raised or normal.

Both alternatives 1 and 2 would imply that the body had no power of synthesising the substance. In No. 1 the cyclose would have simply been washed out of the body by the polyuria, in No. 2 the body would have retained a certain amount, possibly as a necessary constituent of the internal environment of the cells. In both alternatives 3 and 4, however, synthesis would have taken place. Alternative 3 would indicate that no great quantity of cyclose was necessary for the body and that only enough was being synthesised to excrete. The fourth alternative, however, would seem to show that the body does indeed require it and can produce sufficient, not only for the demands of the inosituric condition, but also for its own uses.

The fourth alternative was the one found. This result has some bearing on the views which have been held concerning the general significance of the cyclose group. The only worker to hold a general position on the matter was Starkenstein [1909], who considered that inositol possessed "no special physiological significance" and subserved no important function in the body. It entered, he thought, in the form of phytin obtained from plants, and when the phosphorus was split off from it in the tissues, simply remained there as an inert constituent. Thence it was merely washed out in polyuric states. If, however, this was the case, the first alternative would have been expected.

The inositol stored by accident, as it were, in the rats' tissues would have been washed out and after a time the excretion would have stopped when the animal had become cyclose-free. In actual fact, the contrast to this was marked. A real synthesis undoubtedly took place, and Starkenstein's theory becomes accordingly difficult to hold. The fact that synthesis does take place is, of course, no proof that inositol has any metabolic significance, for it might owe its existence to the chance presence of its precursors. But substances which possess physiological significance are surely more likely to be synthesised by the animal body than substances which have no part to play in metabolism.

Summary.

1. There is no change in the amount of free inositol contained in the tissues of the rat after eight months on an inositol-free diet.

2. Polyuria and inosituria artificially induced in rats by feeding them on a salt diet over a period of 110 days, caused a lasting and vigorous excretion of inositol, without any diminution in their body-content, although the diet was inositol-free.

3. The animal body appears to possess the power of synthesising inositol.

The author wishes firstly to express his sense of indebtedness to Professor F. G. Hopkins for his continual inspiration and encouragement. He desires also to thank Mr J. B. S. Haldane and Mr John Freeman; the former for his help as an experimental subject and the latter for carrying out the histological work. Finally he would thank Dr Graham-Smith for his advice concerning bacteria in urine.

REFERENCES.

Anderson (1916). *J. Biol. Chem.* **25**, 401.
Bürger (1873). *Deutsch. Arch. klin. Med.* **11**, 343.
Cloetta (1856). *Liebig's Annalen*, **99**, 302.
Cole (1919). Practical Physiological Chemistry (Heffer and Sons).
Danileffski (1880). *Ber. deutsch. Chem. Ges.* **13**, 2132.
Ebstein (1873). *Deutsch. Arch. klin. Med.* **11**, 352.
Fowler and Hawk (1910). *J. Exp. Med.* **12**, 388.
Hopkins (1923). Private Communication to the author.
Gallois (1864). *Thesis*, Paris.
Graham-Smith (1924). Private Communication to the author.
Greenwald (1917). *J. Biol. Chem.* **31**, 1.
Klein (1909). *Dissert.* (Giessen).
Külz (1875). *Med. Centr.* Maly, 131; (1876) 124 and 45.
—— (1876). *Sitzber. Ges. Naturwiss. Marburg*, **4**.
Leva (1891). *Deutsch. Arch. klin. Med.* **48**, 173.
Mayer (1907). *Biochem. Z.* **2**, 393.
McCollum and Hart (1908). *J. Biol. Chem.* **4**, 497.
Meillère (1906). *Compt. Rend. Soc. Biol.* (2), 159.
—— (1907). *Compt. Rend. Soc. Biol.* **1096**.
Momose (1916). *Biochem. J.* **10**, 120.
Mosler (1868). *Virchow's Arch.* **43**, 229.
Müller (1907). *Sitz. Naturforschergesel. Rostock*, **19**

Needham (1923, 1) *Biochem. J.* **17**, 422, 431.
—— (1923, 2). *J. Physiol.* **57**, *Proc.* lxxx.
Neukomm (1860). *Dissert.* Zürich.
Offergeld (1906). *Z. Gebürtsh. Gynäkol.* **58**, 189.
Plimmer and Scott (1909). *J. Physiol.* **38**, 247.
Przibram (1871). *Vierteljahrsch. prakt. Heilk.* **112**, 1.
Roberts (1865). Urinary and Renal Diseases.
Rosenberger (1908). *Z. physiol. Chem.* **56**, 373; **57**, 464.
—— (1909). *Z. physiol. Chem.* **58**, 369.
—— (1910). *Z. physiol. Chem.* **64**, 341.
Starkenstein (1908). *Z. physiol. Chem.* **58**, 162.
—— (1909). *Z. exp. Path. Therap.* **5**, 378.
—— (1911). *Biochem. Z.* **30**, 56.
Vohl (1858). *Arch. Physiol. Heilk.* **17**, 410.

CXIX. THE RATE OF REPRODUCTION IN ARTIFICIAL CULTURE OF *COLPIDIUM COLPODA*. PART III.

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The rate of growth and the number of divisions a unicellular organism undergoes in a given period of time has attracted the attention of biologists for many years. Numerous publications by bacteriologists have appeared on this subject; and the work of Maupas [1888], followed later by that of Woodruff, Calkins and others, has brought it into prominence as regards protozoa.

In 1923 we published the results of certain experiments on the ciliate *Colpidium colpoda*, our object being to show the necessity of keeping experimental conditions rigidly constant if comparable results were to be obtained. Among other variable factors the food supply was considered. It was realised that this was a factor particularly difficult to control, owing to the fact that the culture of the ciliate was contaminated by a species of a small bacillus. This was extremely difficult to exclude from the culture; and, moreover, when its numbers were reduced the ciliates showed typical signs of hunger degeneration, making it impossible to carry sub-culturing further. To obviate the difficulty, therefore, we decided to give to each sub-culture, either at the time of inoculation, or after 24 hours' growth, a plentiful supply of an easily recognisable species of bacterium, *Sarcina lutea*, in the belief that so long as plenty of these were present the food supply would be more than adequate to meet the requirements of the protozoa. Our experimental results appeared to justify this assumption, which also received support from the work of other observers, who had worked on a similar basis. Further, as the ciliate we were using was of the same strain as that used by Peters [1921], for his work on the growth of protozoa without bacteria, the possibility of deficient bacterial food supply seemed remote.

Recently, however, we have had to revise our views for the following reasons. Experiments were started to test which of the two bacteria, the contaminating bacillus or the added Sarcina, provided the better food supply. This involved counting the numbers of bacteria and protozoa in the cultures; whereupon it at once became evident that there was a very definite relationship between the number of divisions of the ciliate and the numbers of bacteria present.

The original experiment was therefore discontinued and new ones started. Here the procedure was to count the numbers of bacteria (the "contaminating" bacillus alone being used, because it seemed to be more acceptable to the ciliate than was Sarcina) and the numbers of protozoa at 24-hour intervals until the protozoa had attained their maximum numbers. In order to reduce the results to a common basis the bacterial numbers were expressed as a ratio, obtained by dividing the figure for the protozoa into that for the bacteria at each counting period. The ratio thus obtained gives the number of bacteria for each individual protozoan. In Table I the number of divisions for 24-hour periods are grouped together against the bacterial ratios which are also grouped as a geometrical progression. It is seen that the number of divisions increases as the number of bacteria per individual protozoan increases. When the ratio is as low as 500 little or no reproduction takes place, but at a ratio of 1,024,000 the number of divisions becomes as great as 5·3.

Table I.

Ratio	Reproductive rate for 24-hour periods	Average
250	0	0
250–500	0, 0, 0·2, 0·1, 0, 0, 0	0·04
1,000	0·1, 0·4, 0·04, 0·1, 0·03, 0, 0, 0	0·08
2,000	0·1, 0·2, 0, 0·6, 0·1, 0, 0, 0, 0	0·11
4,000	0, 0·1, 0, 0·2, 0, 0·6, 0·3, 0·8, 0·5, 0·4, 0, 0, 0·4, 0, 0, 0, 0·8, 0·6, 0, 0, 0·3, 0·2, 0, 0·4, 0, 0·6	0·24
8,000	0·4, 0·2, 1·4 0, 1·1, 0·7, 0·8, 0, 0·3, 0, 0·5, 0·6, 0, 0·6, 0, 0·2, 1·0, 0·4	0·45
16,000	2·5, 0·4, 1·5, 1·4, 1·3, 0·6, 1·0, 1·1, 0, 0·6, 0·7, 0·5, 0·2, 0, 0·1, 0·7, 0·6, 0·3, 0·9, 0·3, 0·3	0·71
32,000	1·1, 2·1, 0, 0·5, 3·3, 1·0, 0·5, 0, 0·3, 2·1, 2·0, 3·1, 1·2, 2·4, 2·4	1·47
64,000	1·8, 2·0, 1·9, 2·5, 2·9, 3·1, 3·1, 3·1, 2·9, 1·1, 2·7, 1·2, 3·3, 2·7	2·45
128,000	3·4, 3·2, 3·3, 3·4, 3·8, 3·3, 1·8, 2·9, 1·9, 2·3, 2·8, 3·1, 2·1, 2·9, 3·2, 2·7	2·88
256,000	3·2, 4·7, 4·2, 3·6, 4·0	3·94
512,000	4·0, 3·9, 3·6, 3·5, 4·8, 4·8	4·10
1,024,000	5·5, 5·5, 4·9	5·3

It should be pointed out that this is irrespective of the period of time which has elapsed since the culture was started; a culture that has been growing for only 48 hours will give approximately the same number of divisions as one that has been growing for 4 days, provided the ratio in the two cases is the same. For instance, one of our cultures in which, after 8 days, reproduction had ceased and the bacterial ratio was down to 640, was fed with the bacillus so as to raise the ratio to 12,400. Within the next 24 hours 0·9 division had occurred, though the unfed controls showed no reproduction. This phenomenon was repeatedly observed.

Further, it is found that in young cultures the number of divisions in the first 24 hours is dependent on the ratio as the following experiment shows. Cultures were started in which the number of Colpidia was constant, 115 per cc., but the bacterial numbers were varied so that the first series had a ratio of 34,000, and the second, 290,000; in the former the number of divisions was 1·89, but in the second 3·89.

On another occasion when two single cells had been inoculated each into 10 cc. of medium the cultures were fed with bacteria which were then counted,

giving an initial ratio of 18 millions in one case and 4 millions in the other. After 24 hours the numbers of Colpidia were still too low to be counted (under 100 per cc.), but after 48 hours the reproductive rates had been 12·0 and 11·5 respectively, rates which are very much higher than have been obtained, even in 48 hours, in any of the other cultures in which the bacterial ratios have been recorded (see Table I).

This intimate relationship between the bacterial numbers and the reproduction of protozoa probably explains the curious effect which an alteration in the protozoal population of a culture has on the subsequent division. Thus Robertson [1921] has shown that a culture in which reproduction has ceased, can be started again by removing some of the protozoa, the decrease in population being brought about by gently heating. This observation we have confirmed, using gentle centrifuging to remove some of the protozoa.

The most probable explanation of the phenomenon would seem to be that the mere decrease in protozoan population will cause an increase in the bacterial ratio. In the case of heat more ciliates will be killed than bacteria, because of the greater heat resisting power of the latter; while by slow centrifuging the ciliates are carried down, leaving the greater proportion of bacteria still suspended in the supernatant fluid.

Further, the increase in reproduction following inoculation into "bacterised fluid" (that is, culture media in which the bacteria have been allowed to multiply before inoculation with protozoa was made) finds a ready explanation in terms of the bacterial ratio. Robertson [1921] cites one case in which single individuals from two 8 day old cultures were isolated into bacterised fluid and within 24 hours produced 33 and 31 individuals respectively. This is comparable with our experiments, where after feeding with bacteria renewed reproduction took place.

This explanation of course applies only where the bacterial infusion has not been filtered or boiled; it does not therefore cover all Robertson's cases, though here there is the possibility that filtered bacterised fluid may stimulate the growth of bacteria, and so increase the ratio. It is quite possible that the existence of small quantities of X substance is necessary for high reproduction, as asserted by Robertson and others. But it is now evident that regard must also be paid to the action of such substances on the bacteria as well as on the protozoa: for a stimulation of the former will inevitably lead to an increased rate of the latter.

There are two striking differences between Peters' results with *Colpidium colpoda* and our results with the same strain. His cultures very seldom attain to such high numbers as do ours, in fact he considers that 10,000 or more per cc. constitutes an exceptional number of organisms, while ours seldom drop below this number and are generally considerably above it; and, further, this maximum is reached very much later, after about 12 days as against 6 days or less in our cultures. These discrepancies are readily explained on the supposition that Peters' cultures were contaminated with the same bacillus

that occurs in ours, but that he had reduced their numbers to a minimum. Such being the case the ratio would be very low from the beginning of every sub-culture, and the subsequent growth of the Colpidia would be slow, as the bacteria would have no opportunity to make headway and raise the ratio to a satisfactory figure.

Size of inoculum.

In previous papers it was stated that the variation in the number of animals inoculated, within limits (100–700), appeared to bear no relation to

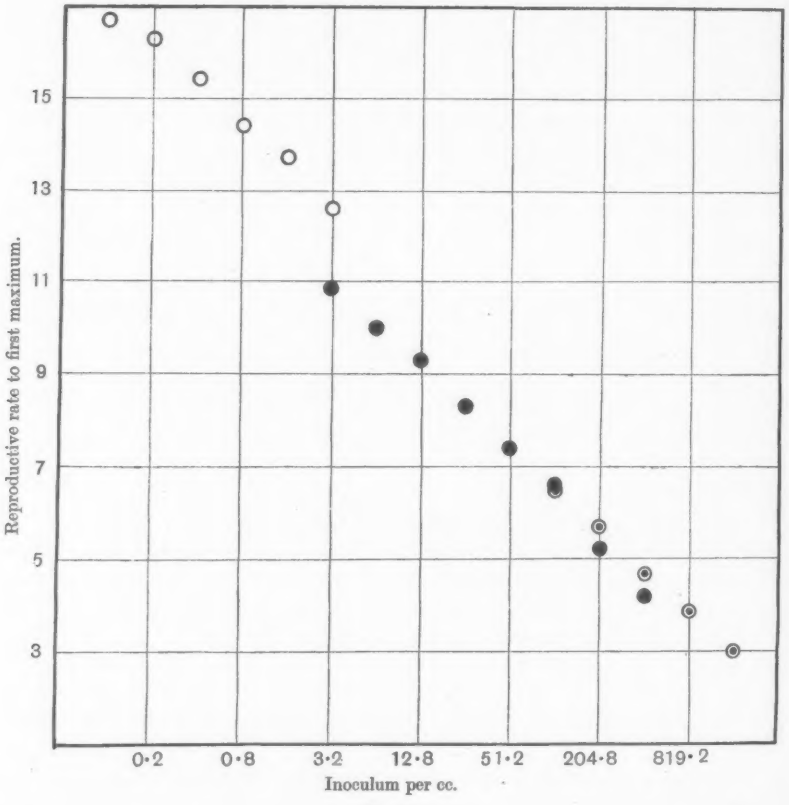

Fig. 1.

○ 1–3 animals in 0·25 to 10 cc. ● Mass cultures inoculated with 4–820 animals per cc.
⊙ 1–3 animals in less than 0·005 cc.

the subsequent growth of the culture, except in the few cases where single cells were inoculated into 0·25–1·0 cc. That is to say, that the reproductive rate is practically constant, lying between 5·0 and 6·5 divisions. Later work

has shown that this statement is misleading. If all the results are ranged in ascending order according to their inocula, beginning from one animal in 10 cc. and ending at 1600 per cc., and the inocula are then grouped in geometrical progression the reproductive rates fall steadily, the average for each group being greater approximately by one than that for the succeeding group (Fig. 1). The cultures whose results are shown in Fig. 1 were all grown in the same medium, and in the great majority of cases are derived from 1 or 2-day parents; they fall into three groups according to the method by which they were made. Those with the lower inocula, that is, from 0·1 to 4 per cc. (groups 0·1–0·2 to 3·2–6·4), came from single cells isolated into comparatively large quantities of medium, 10 cc. being the largest amount used and 0·25 cc. the smallest.

The second group of results shown in Fig. 1 come from mass cultures made in the ordinary way; that is, the number of cells in a stock culture are counted, and the amount of this culture that will give a suitable concentration in 10 cc. of fresh medium is calculated, and taken out in a sterile pipette. The inoculum in the mass cultures varies from 4 to about 500 per cc. Thus an inoculum of 4 or its equivalent per cc. occurs both in the first and second groups, in one case by the inoculation of one animal into 0·25 cc., in the other by inoculating 40 into 10 cc. The average reproductive rate is, however, different in the two cases, the single cell cultures giving a higher number than the mass cultures. It is possible that this discrepancy is due to the fact that in the mass cultures the number inoculated is not of necessity accurate, but it seems more likely that there is some real difference, as a line drawn through all the points of the first group is on a higher level than one drawn through those of the second, showing that the difference is common to all the points concerned.

The third group of results is derived from single cells grown in counting-chambers [Cutler and Crump, 1923]; here the inoculum is in every case a single cell, but, as the volume of liquid is very small, it is equivalent to a high concentration per cc. The points in this group fall very well into line with those of the second group.

The conclusion to which these results obviously point is that each culture tends to arrive at a constant maximum population per cc. irrespective of the number of animals from which it starts. That earlier work suggested a constant reproductive rate between certain limits was due to the fact that the limits were comparatively narrow and that relatively few results were available. In Table II all the results of Fig. 1 are given, together with the coefficient of variation and range of each group, and in this case all the cultures are massed together regardless of the way in which they were made.

As has been pointed out before [Cutler and Crump, 1923], Robertson [1922] found considerable difficulty in making successful cultures of *Enchelys* from single cells in 0·1 cc., or more in volume, and, further, stated that this was a common experience. A review of the literature bearing on this point, however, indicates that both bacteria and protozoa can be isolated into very

Table II.

Inoculum per cc.	Standard deviation	Range	Average reproductive rate	No. of cases
0·1–0·2	0·405	1·5	16·7	16
0·2–0·4	0·705	2·2	16·3	8
0·4–0·8	0·389	1·1	15·4	8
0·8–1·6	0·545	1·8	14·4	13
1·6–3·2	0·511	1·7	13·7	12
3·2–6·4	1·166	3·7	11·6	19
6·4–12·8	0·701	2·7	10·0	10
12·8–25·6	1·130	2·9	9·3	12
25·6–51·2	0·520	1·8	8·3	14
51·2–102·4	1·233	3·4	7·4	14
102·4–204·8	0·619	2·6	6·6	25
204·8–409·6	0·823	2·4	5·5	32
409·6–819·2	0·719	3·0	4·7	17
819·2–1638·4	1·334	4·2	3·9	10
1638·4–3276·8	1·229	3·7	3·0	8

much greater volumes without impairing their reproductive activity. Thus Barber [1908] has grown single bacteria in 5–10 cc. of broth and records that his failures are probably due to faulty technique; Churchman and Kahn [1921], working with *B. coli*, found that in 80 % of the cases single bacteria would grow, presumably in large quantities of broth, though the exact amounts are not stated. In the case of protozoa, Balbiani [1860] asserted that single Paramoecia require 2–3 cc. of infusion for the greatest reproduction to take place; Woodruff [1911] found that Paramoecia in 40 drops divided 7·4 % times more rapidly than those in 2 drops, and Taliaferro [1923] claims to have infected rats with single specimens of *Trypanosoma lewisi*.

The results obtained in our work bear out Barber's contention that failure of single cells to grow in large volumes of liquid is due to imperfect technique, and further indicate that speed in transferring the isolated animal from the parent culture to the new medium is the most important point to be observed.

From the point of view of the bacterial ratio the single cell cultures in 10 cc. of medium form an extreme case which should be of interest. In all, 16 of these cultures have been grown and in every case the parent from which the single cell came was 24 hours old. The first set contained four parallels and was unfed, the final average reproductive rate was 16·4, and the maximum number of Colpidia was attained on the 8th and 9th days after inoculation. There were six parallels in the second set, again unfed; here the average reproductive rate was 17·0 and the maximum numbers occurred on the 5th and 6th days. In all the other cases the cultures were fed with the bacterium; in four of these the average content of bacteria after 24 hours was 87 millions; in the other two, put up on another occasion, the average content after 24 hours was 180 millions. Of these six cultures, all reached their maxima on the 3rd or 4th days after inoculation, in the first case with an average reproductive rate of 16·9 and in the second of 17·3. The effect of the bacteria is very marked in these cases in speeding up the growth of the Colpidia, although the final reproductive rate varies very little.

One of the great difficulties encountered in drawing deductions from the behaviour of organisms grown in artificial cultures is that the medium changes rapidly by the formation of waste products, whereas, under natural conditions, the medium would not accumulate these to any marked degree. It was thought that a culture might be freed to a considerable extent from the products of metabolism and, at the same time, be kept fairly constant by growing it inside a permeable membrane, surrounded by medium which could be frequently changed. For this purpose collodion sacks were made in the way suggested by Gates [1921]; that is, by dipping a gelatin capsule into collodion and later washing out the gelatin with hot water. If these are made carefully the collodion remains permeable even after autoclaving, though it shrinks to some extent during the process. Such sacks, containing 10 cc. of medium and surrounded by about 80 cc. of the same liquid, were inoculated with Colpidia in the ordinary way with most surprising results. Not only was the reproductive rate higher after the first day or two, but from the first count the ciliates in the membrane were very much larger than those in the control culture in a flask. Table III shows results from three typical cultures:

Table III. *Reproductive Rate to first maximum.*

Inoculum	Control	Membrane
40 per cc.	7·7	10·8
80 „	6·7	10·9
115 „	6·5	9·5

When the effect of the bacterial ratio was first noticed a count was made on a culture in a membrane and in a flask, when the number of bacteria proved to be very much higher in the membrane, even to the extent of forming a dense white deposit which made accurate counting impossible. The high results obtained from cultures made in these permeable membranes therefore seem to be due to the fact that bacterial growth is stimulated, probably by the provision of food substances contained in the sack. Such substances are derived from the gelatin capsules on which the sacks have hitherto been made, as the addition of collodion to a culture has no effect on its growth, while if a sack made up on gelatin in the ordinary way and then cut up is added to a flask culture the growth is once more increased.

Summary.

Experimental evidence is given that the number of divisions *Colpidium colpoda* undergoes in definite periods of time is intimately connected with the size of the bacterial population.

Further investigations on the relation between the size of the inoculum and the rate of reproduction demonstrate that the number of divisions steadily decreases as the number of animals inoculated increases.

REFERENCES.

Balbiani (1860). *Compt. Rend. Acad. Sci.* **50**, 1191.
Barber (1908). *J. Infect. Dis.* **5**, 379.
Churchman and Kahn (1921). *J. Exp. Med.* **33**, 583.
Cutler and Crump (1923). *Biochem. J.* **17**, 174, 878.
Gates (1921). *J. Exp. Med.* **33**, 25.
Maupas (1888). *Arch. Zool. Exp.* **6**, 165.
Peters (1921). *J. Physiol.* **55**, 1.
Robertson (1921). *Biochem. J.* **15**, 595, 612.
—— (1922). *J. Physiol.* **56**, 404.
Taliaferro (1923). *J. Exp. Zool.* **37**, 127.
Woodruff (1911). *J. Exp. Zool.* **10**, 557.

CXX. NOTE ON THE ESTIMATION OF URIC ACID BY THE HOPKINS-FOLIN METHOD.

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In metabolic experiments where uric acid in the urine is estimated the Hopkins-Folin method is the one most commonly used. In spite of this popularity difficulty is almost universally found in the determination of the end point of the titration with permanganate, and, in addition, disconcerting variations not infrequently occur in a series of parallel estimations.

As long ago as 1876 a method of estimating uric acid in urine was given by Sutton [1876], and, from what is said there, permanganate had evidently been used for estimation of uric acid before that. There it is advised to precipitate uric acid from 300 to 500 cc. of urine by means of 5 to 8 cc. of pure HCl. The precipitation is complete in 24 to 30 hours. The precipitate is then washed once with cold distilled water, dissolved in a solution of pure KOH, and, after acidifying with H_2SO_4, titrated with decinormal permanganate.

In 1887 Blarez and Denigès [1887] drew attention to two factors modifying this titration with permanganate. They found (*a*) that the amount of $N/10$ permanganate required to reach the end point increased as the amount of 20 % H_2SO_4 used in acidifying before titration increased, (*b*) that the degree of dilution of uric acid markedly affected the amount of permanganate required to reach the end point. When the dilution of uric acid was above 1:8000 (from 1:8000 to 1:160,000) there was no effect on the end point, but below 1:8000 (1:8000 to 1:2000) the amount of permanganate required increased as the concentration of uric acid increased. (*c*) That temperature did not affect the end point.

The next stage in the evolution of this method was when Hopkins [1891, 1892, 1893] showed that uric acid could be quantitatively precipitated from urine by saturation with ammonium chloride in two hours. The final estimation was by weighing or, preferably, by titration with permanganate after acidifying with 20 cc. of pure strong sulphuric acid which produced a temperature in the neighbourhood of 60°. This temperature was considered the optimum for titration.

Shortly afterwards Folin [1898, 1901] investigated the method and the instructions given in his second paper are practically those followed at the present day. It was in the second paper that the uranium solution was introduced to precipitate the mucoid bodies in the urine in order to facilitate filtration.

This uranium solution contains 500 g. ammonium sulphate, 5 g. uranium acetate and 60 cc. 10 % acetic acid in 650 cc. water. The resulting volume is about 1 litre. When 25 cc. of this solution is added to 100 cc. of urine the concentration of ammonium sulphate in the mixture is about 10 %.

The method employed in this laboratory is as follows:

100 cc. urine is measured into a tall narrow cylinder and 25 cc. of the uranium solution is added. The mixture is allowed to stand without stirring for about half an hour. The uranium precipitate has then settled and the clear supernatant fluid may be removed by decantation. 100 cc. of this fluid is measured into a beaker, 5 cc. of strong ammonia added and the mixture allowed to stand overnight. The precipitate is then filtered off and washed with 10 % ammonium sulphate solution until chloride-free. The filter paper is removed from the funnel, unfolded, and the precipitate washed back into the beaker with water, the filter paper and water to make about 100 cc. added. The precipitate is now dissolved by the addition of 15 cc. concentrated H_2SO_4 and, at once, whilst warm, titrated with $N/20$ potassium permanganate. The end point is the first appearance of a permanent pink colour. A correction of 2·4 mg. due to the solubility of ammonium urate is added to the result.

Folin [1901] states that uric acid exposed to a solution of ammonia is destroyed, but in the presence of 10 % ammonium sulphate no destruction occurs after 48 hours' exposure. The addition of the filter paper to the 100 cc. water before adding the strong sulphuric acid is not advised by Folin as he considers the strong acid may destroy the paper liberating substances capable of reducing the permanganate solution. The optimum temperature [1898] for the titration is given as lying between 55° and 65°. Below 50° the end point is not sharp and above 70° the colour disappears too rapidly. The correction factor of 2·4 mg. of uric acid for 80 cc. of urine is obtained from Folin's calculation that the solubility of this acid in urine is about 3 mg. per 100 cc. urine. Also it is stated by Folin that traces of chloride do not disturb the titration.

In the following estimations a standard solution was made by dissolving the uric acid by the aid of Li_2CO_3 in water. The addition of a trace of thymol prevented destruction for several weeks.

An obvious source of error is the presence of reducing substances either in the water or in the concentrated sulphuric acid used.

Glasgow (Loch Katrine) water, which was used throughout, contains 0·80 parts per 100,000 of organic matter and absorbs 0·16 part of oxygen per 100,000 from permanganate when exposed for 4 hours at 80° F. Nevertheless it was found that one drop of $N/20$ $KMnO_4$ gave a lasting pink colour to 100 cc. water at all temperatures.

With the "pure" sulphuric acid it was quite otherwise. Varying quantities of concentrated sulphuric acid were added to 100 cc. water and titrated at 40° with $N/20$ permanganate. The end point was taken when the pink coloration persisted just beyond10 seconds. The following results are each the average of at least three readings (Table I).

Table I.

cc. concentrated H_2SO_4	1	5	10	15
cc. $N/20$ $KMnO_4$ used A	0·08	0·15	0·235	0·32
„ „ B	0·045	0·07	0·085	0·115

Many other samples of "pure" sulphuric acid were obtained and all showed similar variations.

Sample *B* was then used in estimating uric acid, e.g. as in Table II.

Table II.

cc. concentrated H_2SO_4	1	5	10	15
cc. $N/20$ $KMnO_4$ used in titration	1·355	1·38	1·385	1·42
Corrected figures	1·31	1·31	1·30	1·305

This, then, is a possible explanation of the error observed by Blarez and Denigès, and also of the discrepancies in a series of estimations if no blank titration is performed as a routine.

By varying at one time the solvent and at another the solute, the effect of the degree of dilution of uric acid on the permanganate was investigated (Table III). The conditions of titration were as above, the temperature being raised or lowered to 40° and a correction factor for H_2SO_4 used.

Table III.

Variation in solvent.

Dilution	1 : 20,000	1 : 15,000	1 : 10,000	1 : 8,000	1 : 6,000	1 : 4,000	1 : 2,500
cc. $N/20$ $KMnO_4$	2·615	2·595	2·575	2·575	2·575	2·615	2·625

Variation in solute.

Dilution	1 : 20,000	1 : 10,000	1 : 6 666	1 : 5·000	1 : 2,000
cc. $N/20$ $KMnO_4$	2·60	2·575	2·591	2·583	2·588

Many other series gave similar results. This does not substantiate the findings of Blarez and Denigès that concentration of uric acid plays an important rôle. In this connection it is interesting to note that, from his figures, Folin must have been working at times with a dilution of only 1:1000. In ordinary metabolic work also, the dilution of uric acid from normal urine in the mixture on titration can but rarely be above 1:8000.

The effect of temperature on the end point of the titration is as follows. Under conditions similar to the above a mixture of 15 cc. H_2SO_4 and 100 cc. water was titrated against $N/100$ $KMnO_4$. The temperature was varied by heating or cooling the mixture after the addition of the sulphuric acid (Table IV).

Table IV.

Degrees centigrade	30	40	50	60	70	80
cc $N/100$ $KMnO_4$	0·46	0·50	0·53	0·53	0·56	0·56

Following on this 1 cc. $N/100$ $KMnO_4$ was added to such a mixture of water and sulphuric acid at different temperatures and the time taken for the colour to disappear noted.

Table V.

Degrees centigrade	30	40	50	60	70	80
Time in seconds	420	150	60	44	32	25

This effect when estimating uric acid is shown below (Table VI):

Table VI.

Degrees centigrade	25	35	45	55	65	75	85
cc. $N/20$ $KMnO_4$ used	2·74	2·74	2·76	2·77	2·78	2·79	2·82
Corrected	2·64	2·62	2·60	2·63	2·61	2·62	2·64

Two points are shown here. First, the titrations should always be at the same temperature. The simplest way to ensure this regularity is to make the fluid suspending the ammonium urate exactly up to 100 cc. and to add exactly 15 cc. strong sulphuric acid. This has the advantage of giving consistently a temperature in the neighbourhood of 60°, the optimum for the titration. Other temperatures, however, are quite possible, especially if the second point is attended to. The essential factor is that the temperature should not vary throughout a series of titrations.

Second, in determining the end point, permanganate should be added drop by drop until the final addition gives a pink diffuse colour persisting just beyond a fixed time. Such a suitable time is 10 seconds. Beginners then have not the unsatisfactory experience of deciding for themselves what is meant by a "persistent pink colour."

Folin's objection to the filter paper does not seem to hold good, at least when using a Green's 1 F paper 9 cm. in diameter. Such a paper was cut into segments ranging from $\frac{1}{16}$ to a $\frac{1}{2}$ of the total area, which were then added to the uric acid solution before the sulphuric acid was added. In numerous titrations the paper did not seem to affect the end point. Nevertheless, it can do no harm to add a filter paper, such as is used in filtering off the ammonium urate, to the blank titration.

The direction to remove all chlorides from the precipitate of ammonium urate by washing with 10 % ammonium sulphate is vague. Apart from the almost practical impossibility of removing the last traces of chloride, and Folin's statement that traces of chloride do not affect the end point, there would seem to be a risk of dissolving an appreciable quantity of the ammonium urate on the filter paper by washing with unknown, possibly large, volumes of ammonium sulphate solution in a vain attempt to remove the last traces of chloride. That sodium chloride in quantities of 15 mg. seems to have no effect is shown in the following table. 15 mg. sodium chloride was found to give a marked precipitate with silver nitrate solution, much more marked than any precipitate obtained in the sulphate washings. Varying quantities of sodium chloride were added to uric acid solution before titration. The results are as follows:

Table VII.

mg. NaCl	0	1	5	10	15
cc. $N/20$ $KMnO_4$	2·53	2·55	2·60	2·60	2·58

The effect of washing with ammonium sulphate solution was another matter. The ammonium urate precipitate was obtained by adding a known volume of standard uric acid solution to 10 % ammonium sulphate solution

made alkaline with strong ammonia solution, S.G. 0·880. After standing overnight the precipitate was collected on a filter paper and washed with varying amounts of sulphate solution. The uric acid in the precipitate and also in the mother liquor plus wash water was then estimated. Corrections by blank titrations were used in all cases (see Table VIII).

Table VIII.

mg. uric acid taken	29	29	29	29
cc. $(NH_4)_2SO_4$ used in washing	—	50	100	200
mg. uric acid in precipitate	27·11	26·96	26·85	26·25
mg. uric acid in mother liquor plus wash water	1·58	1·73	1·95	2·25
Total uric acid recovered	28·69	28·69	28·80	28·50

Other series gave similar results. The sulphate solution then has quite a definite solvent action on the ammonium urate.

The question whether strong ammonia solution could rapidly destroy uric acid in water whilst having no effect in the presence of 10 % ammonium sulphate seemed worth testing. In the first series the amount of ammonia solution used in precipitating the uric acid from the sulphate solution varied and the time of exposure to the alkali remained the same, viz. 48 hours, while in the second series the amount of alkali was fixed, 5 cc. as recommended in the method, but the time of exposure varied (see Table IX).

Table IX.

mg. uric acid taken ...	19·39	19·39	19·39	19·39
cc. ammonia solution added	0·5	2·0	5·0	10·0
Uric acid recovered ...	19·31	19·22	18·82	18·64
mg. uric acid taken ...	19·39	19·39	19·39	
Time of exposure in hours	21	47	96	
Uric acid recovered ...	18·88	18·84	18·66	

Destruction, although present, is slight.

Conclusions.

(1) In this method blank titrations are essential, the blank containing 100 cc. water, 15 cc. concentrated sulphuric acid and a filter paper similar to the one used in collecting the ammonium urate precipitate. This blank should be repeated daily with the materials to be used in the estimation that day.

(2) The degree of dilution of uric acid does not affect the titration.

(3) In gauging the end point a time limit, such as 10 seconds, should be used as suggested above.

(4) The temperature should be the same day by day, the optimum being about 60°.

(5) Traces of chloride do not upset the titration with permanganate.

(6) The amount of 10 % ammonium sulphate solution used in washing the precipitate of urate should be not more than 100 cc. and the worker should continue to use the same amount all through his series.

(7) Strong ammonia solution, even in the presence of ammonium sulphate solution, attacks the uric acid so that the amount of ammonia used and the

time the uric acid is allowed to stand in contact with it should not vary from day to day.

These conclusions show that, in spite of the accuracy of the method, comparable results can be obtained only by observing the same technique day after day. This is all the more important as, in metabolic work, the amount of uric acid in the urine per diem is obtained by multiplying the results of such experiments by at least ten, so that a small alteration in technique may give a result which will appear as a noteworthy variation in total output of uric acid.

My thanks are due to Professor Cathcart for his advice and encouragement.

REFERENCES.

Blarez and Denigès (1887). *Compt. Rend. Acad. Sci.* **104**, 789.
Folin (1898). *Z. physiol. Chem.* **24**, 224.
—— (1901). *Z. physiol. Chem.* **32**, 552.
Hopkins (1891). *Guy's Hospital Reports*, **48**, 299.
—— (1892). *J. Path. Bact.* **1**, 451.
—— (1893). *Proc. Roy. Soc. London*, **52**, 93.
Sutton (1876). Volumetric Analysis (3rd ed.), 259.

CXXI. A NOTE ON THE DURABILITY OF VITAMIN A OF COD-LIVER OIL.

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The durability of vitamins is of great interest in their practical application.

As regards vitamin C, it is known that it has slight durability and that anti-scorbutic leaves or fruits lose their potency when dried. Zilva found [1923] that the destruction of the anti-scorbutic factor is favoured by alkalinity and exposure to air, but that solutions that are acidified and stored under anaerobic conditions do not show any perceptible loss in potency after storage for three months.

Interesting observations regarding vitamin B were recently published by Findlay [1923]. He found that certain Indian lentils (*Cicer arietinum, Phaseolus Mungo, Ph. radiatus* and *Ph. Cajanus*) which had been stored in canvas bags in a dry cupboard for at least 38 years, had lost the power of germination, but in daily doses of 3 g. produced adequate growth in rats which had been kept on a diet lacking in vitamin B. 2 g. daily were insufficient. According to Ghose [1922] the same species of lentil, presumably not more than two or three years old, produced growth in doses of 1 g. Smaller doses were not tried. It thus appears that at least two-thirds of potency were lost in those 38 years.

Regarding the fat-soluble vitamin, there exists, as far as I have been able to discover, only one publication [Wright, 1923] to the effect that pork containing 25 % of fat, after being kept for 9 years in a frozen condition, was found in experiments with hens to contain a sufficient quantity of the said vitamin.

A short time ago I received samples of a cod-liver oil which was produced in 1893, and had subsequently been kept in ordinary colourless, practically full glass bottles. It was tried in the usual manner upon rats, which for an adequate time had been living upon a diet that had been freed from vitamin A as far as possible. Daily doses of 2 mg. of this cod-liver oil produced growth the first week only, and later a stoppage or decrease in body weight. The effect of daily doses of 3 and 5 mg. is illustrated by the following curves (only the last week of the preparatory period is included).

The result is thus that this cod-liver oil, 31 years old, in daily doses of 3 mg. caused some growth, in doses of 5 mg. marked growth. It is naturally not possible to have any definite opinion as to whether it had preserved its original

potency. Presumably a part had been lost. In investigations of numerous samples of Norwegian medicinal cod-liver oil at this Institute in the course of the last three years, the great majority were found effective in daily doses of 2 mg., or between 1 and 2 mg., and even one in daily doses of 0·8 mg. (Fig. 2).

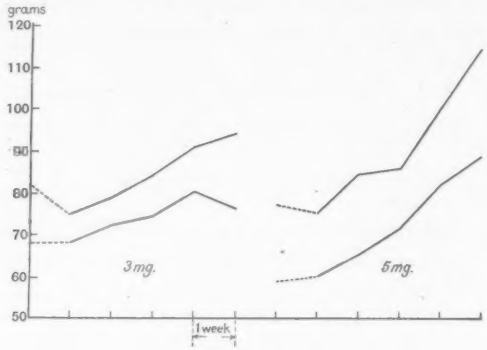

Fig. 1.

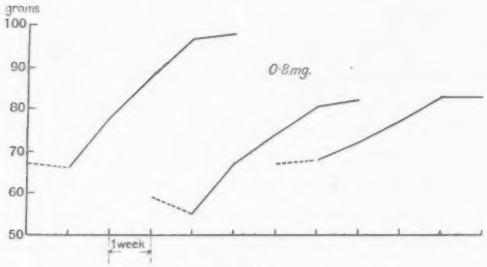

Fig. 2.

REFERENCES.

Ghose (1922). *Biochem. J.* **16**, 35.
Findlay (1923). *Biochem. J.* **17**, 887.
Wright (1923). *J. Soc. Chem. Ind.* **42**, 509, quoted from report in *Chem. Zentr.* 1923, i, 1403.
Zilva (1923). *Biochem. J.* **17**, 410, 416.

CXXII. THE CALCIUM CONTENT OF THE BLOOD SERUM IN CASES OF GOUT.

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The concentration of the inorganic salts of the blood serum in cases of impaired metabolism such as gout, seems to have attracted very little attention although there have been estimations of the urinary calcium under such conditions. Thus Little [1909] examined the urine of a gouty patient both during an acute attack and in a quiescent interval with the result that he found a calcium content of 0·067 g. during the acute period which amount rose to 0·122 g. during the quiescent stage. His calculations are for CaO. He quotes no figures for normal people. Van Noorden [1907] makes no reference to the urinary calcium output in gout cases.

Of investigations of the inorganic salts of the blood sera in this disorder we can only find one recorded examination. Lyman [1917] found a calcium content of 6·0 and 5·7 mg. per 100 cc. of serum in two cases of gout. His normal cases were also approximately 6·0 mg. %. Records of investigations in pathological conditions possibly allied to gout have been made by Weil, Guillaumin and Weismann-Netter [1923]. In an extensive study of the inorganic salts not only did they estimate the calcium in rheumatic cases but at the same time recorded observations on cases of atheroma and of hypertension without atheroma. These findings appear to us so important that we have abstracted their results, taking the average readings of their examinations.

Table I.

Type of case	No. of cases	Average Ca in mg. %
1. Normal	3	10·90
2. Hypertension without atheroma	9	11·02
3. Atheroma	10	13·08
4. Chronic rheumatism	11	14·30

We have therefore thought it advisable to undertake an investigation into the inorganic salts of the blood serum in undoubted cases of gout and the present short paper deals with the calcium findings. We chose the calcium because in the first place it appears to have been almost entirely neglected, secondly because calcium has been found to be a constituent of gouty tophi, and thirdly because cases of long standing gout in some instances show gross

bony lesions. The cases investigated were carefully selected by one of us (V. C.) at the Royal Mineral Water Hospital, Bath, the greatest care being taken to ensure that they were cases of true gout. These cases fell into two classes, viz:

1. Patients from whom it was possible to obtain a clear history of typical arthritic attacks and who exhibited tophaceous deposits, but who at the time of veni-puncture were in a quiescent stage, and

2. Patients seen within thirty-six hours of an acute attack but who had received no treatment.

The blood was collected by veni-puncture from the median basilic vein and allowed to clot in sterile tubes. The resulting serum was centrifuged, kept in the ice-chest and examined as soon as possible. The method of estimating the calcium was that described by Kramer and Tisdall [1921], the amount of serum used being 1 cc. Duplicate determinations were done in all cases where it was possible. The figures we obtained are given below:

Table II.

Subject	Age	Ca content in mg. %	Remarks
A. H.	46	16·4	Interval
H. J. S.	48	22·6	,,
B. P.	52	16·4	Acute
J. S.	53	20·0	Interval
W. A.	63	18·4	,,
H. L.	48	16·8	Acute
		24·0	Interval
G. L. T.	51	16·0	,,

Average of eight determinations = **18·87** mg. %.

An examination of the above figures will show that we have obtained a figure for the calcium of a very much higher order than either Lyman [1917] in gout or Weil [Weil *et al.* 1923] in rheumatism. This increase in the calcium might be due to several causes such as:

1. Variations from the normal of no pathological significance.

2. Increasing age.

3. Hypertension with or without atheroma, both of which conditions commonly accompany gout.

4. Decolorisation of the permanganate by bodies other than calcium, thereby obtaining a high figure.

The first cause can be dismissed in a few words. The calcium of the blood serum of mammals seems to be one of its steadiest constituents. Thus Meigs, Blatherwick and Carey [1919] found a concentration in cows of 9·7 mg. %. Rona and Takahashi [1913], working with horse serum, report 12 mg. %. Watanabe [1918] finds the percentage calcium in the rabbit to vary from 10·5 to 12·8 mg., whilst Boggs [1908] mentions 10 mg. for the dog, and Kramer and Tisdall [1921] give 9·8 to 10 mg. for the human adult. It is therefore apparent that the adult mammal keeps a very constant level of blood calcium and in face of the observations quoted above it seems to us a fair assumption

that the variation from the normal that we have observed has some biochemical significance.

The second cause—advancing age—appears at first sight to be a possible one, but to that view there is, in our opinion, two objections. The first is based upon two recent observations on two people whose ages were 57 and 52, but who were free from, and had never suffered from, gout. Their calcium content was 10·2 and 14·0 mg. % respectively. The second objection is based upon the figures of Weil given above. There we have quoted figures for cases of hypertension and atheroma, and although he has unfortunately omitted to give the age of the patients, yet atheroma, at least, is not usually associated with youth, and we think it a fair assumption that the ages do not differ materially from those of our own gout cases. Assuming this to be so, an inspection of his table will show that his average for hypertension is only 11·02, and for atheroma 13·08, figures far below the average of our gout cases.

The reasons above stated apply with equal force to a consideration of the point whether the rise is due to any condition of atheroma or hypertension. As both these conditions are commonly associated with gout we have given the matter considerable attention but in view of the figures quoted above we feel unable to conclude that so large a rise in the calcium content as we show can be due, entirely, to either cause.

The fourth possibility, viz. that the permanganate is decolorised by some body other than the calcium is worthy of a good deal of consideration. Uric acid and urates present in the serum might account for some of the permanganate. To test this point a determination on the serum of G. L. T. in Table II has been made. Taking the same serum 1 cc. of a solution of sodium urate, containing 0·5 g. in 500 cc. of water, was added to 1 cc. of the serum and the calcium content of the mixture ascertained by the same method. The result was 15·8 mg. % against 16·0 mg. % for the same serum without the added urate. This figure, allowing for experimental error, seems to us to be of the same value and we conclude that within moderate limits, the urates are not affecting the result.

Clark [1921] has pointed out the difficulty of removing all the adsorbed oxalates, and also the impossibility of performing unlimited washings owing to the solubility of calcium oxalate. As oxalates will decolorise permanganate there is some force in the argument that imperfect washings will produce a high calcium value. But in this connection we would like to point out that we have adhered strictly to the method of Kramer and Tisdall [1921] and that they themselves by that method arrive at a normal value of approximately 10 mg. %. If, by the same method, we arrive at a higher figure in certain pathological cases we feel justified in the assumption that any error due to adsorbed oxalates or any other cause will be of approximately the same order, and the increase that we find may justifiably be credited to the calcium.

For all these foregoing reasons we have ventured to say that the calcium

content of serum in cases of gout is considerably raised. The causes and significance of this elevation we have not presumed to discuss as the investigation is still proceeding, and we hope in the near future to be able to publish results of the concentration of other inorganic salts in cases of this obscure metabolic disorder.

It is worthy of note that in one or two recorded cases of hyperplasia of the parathyroids there has been atheroma accompanied by calcification of tendons, etc. [Barker, 1922] Also de Wesselow [1923] has noted a case considered clinically to be one of hyperplasia of the parathyroids in which the calcium content of the serum reached a very high figure.

We have to express our thanks to Dr Gibson of the Radcliffe Infirmary for kindly supplying us with the blood of two non-gouty patients.

Conclusions.

1. The examination of the calcium content of the blood serum in cases of gout shows a considerable rise from the normal.

2. A short discussion attempts to show that this rise is specifically due to the gout and not to any extraneous factor.

REFERENCES.

Barker (1922). Endocrinology and Metabolism, **1**, 702.
Boggs (1908). *Bull. Johns Hopkins Hosp.* **19**, 201.
Clark (1921). *J. Biol. Chem.* **49**, 487.
Kramer and Tisdall (1921). *J. Biol. Chem.* **47**, 475.
Little (1909). *Biochem. J.* **4**, 30.
Lyman (1917). *J. Biol. Chem.* **30**, 1.
Meigs, Blatherwick and Carey (1919). *J. Biol. Chem.* **37**, 1.
Van Noorden (1907). Metabolism and Pract. Medicine.
Rona and Takahashi (1913). *Biochem. Z.* **49**, 370.
Watanabe (1918). *J. Biol. Chem.* **36**, 531.
de Wesselow (1923). *Quart. J. Med.* **16**, 351.
Weil, Guillaumin and Weismann-Netter (1923). *Compt. Rend. Soc. Biol.* **88**, 732.

CXXIII. THE CALCIUM METABOLISM OF ATROPHIC INFANTS AND ITS RELATIONSHIP TO THEIR FAT METABOLISM.

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(*Received July 25th, 1924.*)

For the purpose of this investigation, an infant is considered to be suffering from simple atrophy or marasmus if the body-weight is less than 80 % of the expected body-weight for its age, and if there is no other evidence of disease.

Many theories have been put forward from time to time as to the cause of atrophy, scientific basis for which has not been fully established. Czerny, as is well known, assumes that atrophic children have a low assimilative power for fat, and, as a result of fermentation and digestion, fatty acids are formed in excess in the intestinal tract. To neutralise these fatty acids, alkalies are taken from the tissues and excreted into the intestine and lost in the stool. In constipation, with soap stools, calcium, and in diarrhoea potassium and sodium are thus excreted. Thus, in his view, demineralisation of the body is the essential cause of atrophy.

Holt [Holt, Courtney and Fales, 1920] believes that there is no evidence of demineralisation of the body owing to low assimilative power for fat, and that excessive ingestion of calcium does not result in excessive excretion of calcium accompanied by a harmful loss of fat. He believes that an ample intake of both fat and calcium produces only beneficial results.

Marriot [1920, 1921] believes that atrophy is essentially the result of partial starvation for long periods of time, and that there is a negative mineral salt balance.

It has been shown recently that fat absorption in infantile atrophy (in the absence of diarrhoea) is normal [Parsons, 1924, 1]. The relationship between the fat and calcium metabolism in normal children has been the subject of considerable investigation, but until recently there have not been any extensive observations on the calcium metabolism of definitely atrophic children with the exception of a series of seventeen cases reported by Utheim [1922].

The present series of observations on the calcium metabolism of atrophic infants was made at the request of Dr Leonard G. Parsons, Physician to the Children's Hospital, Birmingham. The fat metabolism was investigated at the same time, so that the relationship between the fat and calcium could be

established. Certain observations made during this investigation have led to the belief that a large number of cases must be observed before any definite conclusions can be drawn. The total number of children investigated was forty-eight, but some observations on thirty-nine of these have already been published [Parsons, 1924, 2].

Preliminary observations showed that there is considerable variation in the composition of stools from day to day, and even from hour to hour; it was therefore deemed advisable to make the period of observation five days if possible. Since the length of time taken by food to traverse the alimentary tract of atrophic children may be as long as three days, to ensure that the stools examined in this series should be those resulting from a known intake of fat and calcium, the following routine was observed [Parsons, 1924, 1].

Collection of specimens.

The infant was given the specified diet for three whole days before the stools were collected, as well as during the period of collection. Since milk varies in composition from day to day, and since the proportion of fat and calcium depends on whether the milk has been properly mixed before use, and also to eliminate any errors due to inaccurate dilution of the milk or milk powder when making up the feeds, half an ounce extra was prepared for each meal and these samples were put together and analysed for fat and calcium every 24 hours for five days. The actual food intake was also observed and any food vomited was noted and subtracted from the intake. There was little vomiting in the cases observed. At the end of the third 24 hours, the infant was placed on a metabolism bed, and stools and urine were collected separately until the end of the eighth day. Every effort was made to collect complete specimens. The 24-hours collections of stools were then dried and analysed for fat and calcium. The mean of the five 24-hours samples of milk was taken as the average daily intake and the mean of the five 24-hours collection of stools was taken as the average daily output.

None of the infants was suffering from diarrhoea during the period of observation.

Methods of Analysis.

The stools were transferred as quickly as possible from the receiver to small glass dishes of known weight, and were then carefully dried for 24 hours (or longer if the stools were large) over an electric heater, thus ensuring a moderate temperature of about 60° to 80°. They were weighed before being pulverised. If the stools were very solid they were mixed with a little water and spread over the dish before being dried. If very fatty, instead of being pulverised, they were treated with ether and stirred thoroughly while the ether evaporated to ensure complete homogeneity, care being taken that there should be no loss during these operations.

In many records published on the calcium metabolism of children, the

observations have been made on a few cases only. Holt, Courtney and Fales [1920] have, however, published a very complete record of the metabolism of thirty healthy children fed on cow's milk, some of whom were having cod-liver oil. In order to compare the results of this investigation with those of Holt and of Utheim, the same method of estimation has been used, namely, that of McCrudden [1920], but bromocresol-purple was used as the indicator in most of these cases; the results are all stated in terms of calcium oxide.

The calcium excreted in the urine of all the cases observed was not estimated, so that the figures do not show the actual retention of calcium. The retention is, however, not very different from the absorption. Holt states that the daily calcium excretion in the urine of healthy children fed on cow's milk is between 0·01 and 0·05 g. daily. A number of specimens of the urine in this series were examined and a similar range was found, viz. 0·017 to 0·034 g. The calcium excreted in the urine was from 1·8 to 6·0 % of the total calcium excreted, a figure slightly greater than that given by Findlay, Paton and Sharpe [1921], who state that it is less than 4 %; but the high figure of 6 % obtained here was in a case fed on a humanised dried milk.

Many previous investigators have used very different methods for the determination of faecal fat, and there have been differences in the methods of reporting the findings, so that the results are not strictly comparable. Holt, Courtney and Fales [1920] estimated the fat by a modified Rose-Gottlieb method. A method somewhat similar has been used in this investigation. The total weight of the dried faeces for the 24 hours having been found, about 0·5 g. of the pulverised material was weighed into each of two fat extraction tubes. To one was added 10 cc. of 30 % HCl and to the other 10 cc. of distilled water. Both were heated in a boiling water-bath for half an hour, cooled and extracted with 40 cc. of ether by shaking. After settling, 20 cc. of the clear ether solution was transferred with a pipette to a weighed flask of about 50 cc. capacity and evaporated. Repeated experiments under the same conditions, but without the material to be extracted, showed that on the average 3·4 cc. of the ether dissolved in the water or dilute acid, so that the weight of the residue from 20 cc. of the extract must be multiplied by 1·83 to give the total weight of the fat extracted from the known weight of the dried faeces. This eliminated the filtering process which is essential when all the ether extract is evaporated as in the modified Rose-Gottlieb method used by Holt and his co-workers.

This method was tested by estimating the fat in specimens of milk powder by this process, and by a Soxhlet extraction process. The following figures were obtained:

	Soxhlet	Author
(1)	22·87 %	23·19 %
		22·52
(2)	28·63	28·64
		28·47
(3)	30·38	30·84
		29·52

Table I. *Calcium Metabolism.*

No.	Food	% Fat in Food	% CaO in Food	Wt. of D.F. in 24 hrs. g.	% Total ash in D.F.	% CaO in D.F.	Per 24 hours: Fat intake g.	CaO intake g.	CaO output g.	CaO absorbed g.	% Absorption of CaO	Intake of CaO for 1 g. fat	Per kilo. body weight: Fat absorbed g.	CaO intake g.	CaO output g.	CaO absorbed g.
1	Humanised milk	4·9	0·128	17·1	14·4	3·7	41·5	1·10	0·66	0·44	40·2	0·027	8·9	0·29	0·17	0·12
2	,,	4·2	0·113	13·5	15·0	5·0	29·6	0·81	0·66	0·14	17·8	0·027	6·3	0·23	0·19	0·04
3	,,	4·2	0·122	11·4	16·8	5·8	29·1	0·84	0·66	0·17	21·0	0·029	6·5	0·24	0·19	0·05
4	,,	4·2	0·112	4·2	17·6	8·6	30·9	0·83	0·37	0·46	55·6	0·027	7·3	0·21	0·09	0·12
5	,,	3·2	0·094	4·9	22·8	6·9	14·4	0·43	0·34	0·08	19·5	0·030	4·9	0·16	0·11	0·05
6	,,	3·5	0·100	3·8	20·7	9·0	26·2	0·74	0·35	0·39	53·6	0·029	8·3	0·25	0·12	0·13
	Average	—	0·111	9·1	17·9	6·5	28·6	0·79	0·51	0·28	34·6	0·028	7·0	0·23	0·14	0·09
7	Dried milk full-	3·1	0·176	21·5	21·5	7·5	38·3	2·16	1·60	0·56	25·9	0·056	5·0	0·41	0·31	0·10
8	cream	2·1	0·160	5·9	22·1	10·8	19·4	1·50	0·63	0·87	58·1	0·077	3·3	0·32	0·15	0·17
	Average	—	0·168	13·7	21·8	9·2	28·9	1·83	1·12	0·72	42·0	0·067	4·1	0·37	0·23	0·14
9	Dried milk half-	1·4	0·162	4·0	—	15·6	11·5	1·38	0·63	0·75	54·2	0·120	2·5	0·39	0·13	0·26
10	cream	1·3	0·143	2·0	26·3	13·1	7·5	0·73	0·24	0·49	67·3	0·098	3·7	0·40	0·16	0·24
11	,,	1·2	0·120	1·9	30·8	14·6	10·6	1·04	0·26	0·78	75·4	0·099	3·9	0·39	0·10	0·29
	Average	—	0·142	2·6	28·6	14·4	9·9	1·05	0·38	0·67	65·6	0·106	3·4	0·39	0·13	0·26
12	Condensed	2·0	0·086	4·8	13·7	6·0	10·2	0·44	0·29	0·15	34·0	0·044	2·3	0·14	0·09	0·05
13	Milk and water and	3·3	0·147	7·3	27·7	13·6	30·9	1·38	0·99	0·38	27·9	0·044	6·3	0·31	0·22	0·09
14	sucrose	3·2	0·176	7·5	30·1	14·3	29·0	1·63	1·06	0·57	34·8	0·056	5·6	0·35	0·23	0·12
15	,,	3·0	0·150	6·3	16·2	7·3	25·0	1·26	0·48	0·78	61·4	0·050	5·0	0·29	0·11	0·18
16	,,	2·6	0·118	7·7	27·0	11·3	26·1	1·28	0·79	0·40	30·9	0·049	4·4	0·24	0·15	0·09
17	,,	2·4	0·145	7·5	19·7	11·6	19·9	1·23	0·86	0·37	30·3	0·062	3·7	0·27	0·19	0·08
18	,,	2·4	0·119	3·2	26·6	15·4	16·3	0·81	0·48	0·33	40·6	0·049	3·6	0·19	0·11	0·08
19	,,	1·7	0·131	8·9	22·6	5·6	14·7	1·14	0·48	0·66	57·6	0·078	2·9	0·35	0·15	0·20
20	,,	1·6	0·113	1·4	28·8	6·5	10·5	0·75	0·09	0·65	87·6	0·071	2·7	0·20	0·02	0·17
21	,,	*2·6	0·132	3·7	27·5	14·8	18·5	0·88	0·54	0·34	38·7	0·048	5·0	0·26	0·16	0·10
22	Milk and water and	3·1	0·160	6·2	27·6	10·7	26·3	1·36	0·65	0·71	52·2	0·052	5·5	0·31	0·15	0·16
23	dextri-maltose	2·8	0·112	3·9	24·3	9·5	14·5	0·57	0·38	0·20	34·4	0·039	4·9	0·22	0·14	0·08

24	„	2·8	0·103	3·6	24·4	12·5	14·9	0·55	0·45	0·10	18·2	0·037	4·6	0·19	0·16	0·03
25	„	†2·7	0·181	10·8	23·8	12·3	35·3	2·36	1·33	1·03	43·6	0·067	6·2	0·36	0·20	0·16
26	„	*2·7	0·143	2·6	31·6	11·2	16·0	0·87	0·29	0·57	65·7	0·054	5·2	0·30	0·10	0·20
27	„	2·6	0·122	6·6	18·6	9·7	15·7	0·73	0·66	0·06	8·7	0·047	4·2	0·25	0·23	0·02
28	„	*2·4	0·155	8·5	18·2	8·1	24·1	1·42	0·68	0·73	51·7	0·059	5·2	0·42	0·20	0·22
29	„	‡2·3	0·174	7·3	25·1	13·1	16·4	1·22	0·95	0·27	22·2	0·074	5·0	0·44	0·34	0·10
30	„	2·3	0·126	2·4	21·9	11·1	15·7	0·84	0·26	0·58	68·8	0·054	3·6	0·20	0·06	0·14
31	„	2·3	0·144	2·2	25·5	12·6	11·9	0·74	0·26	0·48	65·0	0·040	4·3	0·29	0·10	0·19
32	„	2·3	0·130	7·5	26·6	9·2	21·1	1·22	0·70	0·52	42·8	0·058	4·3	0·28	0·16	0·12
33	„	2·0	0·093	6·7	24·7	11·8	27·5	1·27	0·77	0·50	39·5	0·046	4·2	0·21	0·13	0·08
34	„	2·0	0·111	6·8	17·9	8·7	17·3	0·98	0·59	0·39	39·6	0·057	4·5	0·31	0·17	0·14
35	„	2·0	0·127	6·3	16·9	5·8	12·9	0·81	0·35	0·45	56·0	0·063	3·7	0·29	0·13	0·16
36	„	1·9	0·126	3·1	28·5	12·3	11·9	0·81	0·56	0·25	31·0	0·074	3·7	0·28	0·20	0·08
37	„	1·8	0·117	10·7	18·4	5·6	15·0	1·00	0·57	0·43	42·8	0·066	2·1	0·25	0·14	0·11
38	„	1·8	0·128	3·7	20·8	10·6	11·4	0·83	0·39	0·44	52·7	0·073	3·0	0·24	0·12	0·12
39	„	1·8	0·130	2·0	23·4	5·9	8·1	0·59	0·12	0·47	80·0	0·073	2·8	0·22	0·04	0·18
40	„	1·7	0·108	7·4	23·6	7·2	19·5	1·22	0·52	0·69	57·0	0·063	4·4	0·31	0·13	0·18
41	„	1·7	0·104	4·5	38·1	8·2	9·2	0·56	0·36	0·20	35·2	0·061	2·7	0·19	0·12	0·07
42	„	1·6	0·139	7·7	35·7	12·2	18·0	1·56	0·94	0·63	40·2	0·087	4·1	0·43	0·26	0·17
43	„	1·4	0·101	4·4	21·5	5·0	6·6	0·49	0·22	0·27	55·6	0·074	2·3	0·21	0·10	0·11
	Average	—	0·131	5·8	24·6	10·1	18·1	1·04	0·57	0·47	45·6	0·059	4·2	0·28	0·15	0·13
44	Skim milk and D. M.	1·6	0·146	4·1	38·8	18·6	9·3	0·87	0·77	0·10	12·0	0·093	2·8	0·29	0·26	0·03
45	Skim milk and	§1·1	0·162	1·8	33·3	17·2	13·9	2·03	0·33	1·70	83·7	0·146	2·6	0·40	0·07	0·33
46	sucrose	0·9	0·153	6·4	29·6	13·2	8·6	1·52	0·83	0·69	45·2	0·176	1·6	0·33	0·18	0·15
47	Skim milk and D. M.	0·8	0·127	7·8	29·8	13·1	6·3	0·97	1·03	(−0·07)	0	0·153	1·5	0·28	0·29	(−0·02)
48	„	0·6	0·150	4·4	37·5	19·2	5·1	1·28	0·84	0·43	26·9	0·250	1·0	0·29	0·19	0·10
	Average	—	0·144	5·7	33·9	16·0	7·3	1·16	0·87	0·31	21·0	0·168	1·7	0·30	0·23	0·07
Grand Average (48 cases)		—	0·131	6·3	23·8	10·3	18·3	1·04	0·60	0·44	43·0	0·067	5·3	0·28	0·16	0·12

* HCl added to make p_H 5·2 to 6·0 (approx.).
† Casec added.
‡ Citric acid added to make p_H 5·2 to 6·0 (approx.).
§ Not included in averages.

The milk fat in all the specimen feeds was estimated by a similar method. After heating 10 cc. of the milk with 10 cc. of concentrated hydrochloric acid for half an hour in a water-bath, it was extracted with 30 cc. of ether and a similar correction was made. The residue from the ether extract of the dried faeces after treating with hydrochloric acid is reported as "total fat," but in reality is a mixture of fatty acid from the saponified fat, the free fatty acid and the neutral fat. The ether extract after treating with water only is reported as "unsaponified fat." It is a mixture of free fatty acid and neutral fat. "Total fat" – "Unsaponified fat" gives the saponified fat in terms of fatty acid and is reported as "saponified fat," as in Holt's paper.

The results obtained have been tabulated according to the kind of food, since all the specimens examined were either formed or semi-formed.

Discussion of results.

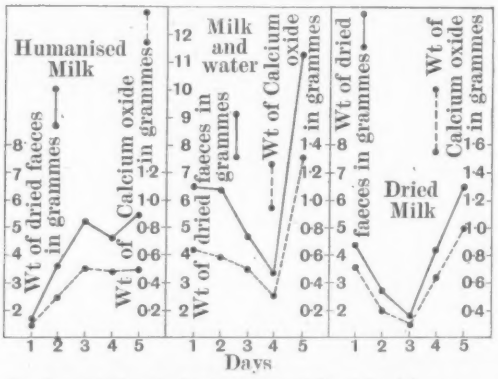
Humanised Milk
Wt of dried faeces in grammes
Wt of Calcium oxide in grammes
Milk and water
Wt of dried faeces in grammes
Wt of Calcium oxide in grammes
Dried Milk
Wt of dried faeces in grammes
Wt of Calcium oxide in grammes
Days

Fig. 1. To show relationship between the weight of dried faeces •——• and the weight of calcium oxide (daily) •· · · · · ·•

Relationship between the Weight of Faecal Matter and the Calcium Output. A consideration of Fig. 1 shows that the daily output of calcium oxide varies fairly regularly with the weight of the dried faecal matter for individual cases. Fig. 2 shows that while on the whole there is diminished output of calcium oxide with diminished weight of faecal matter for all the cases on any one diet, there is a very decided tendency for the lines representing faecal output and calcium oxide to converge as the faecal output diminishes. In other words, the proportion of calcium oxide in the faecal matter tends to become greater as the faecal output diminishes.

Absorption of Calcium. Fig. 3 shows the relationship between intake and output of calcium for the different diets. It is clear that the curves of intake and output of calcium are more or less parallel for any particular diet, though the two lines approach very closely at several points. Where the diet has a low fat content, as in skim milk, the output may be greater than the intake, indicating the output of endogenous calcium. This is in accordance with much

of the modern work on the absorption of calcium. Hopkins [1920] proved that oxidation destroys fat-soluble vitamin A. Making use of this observation, McCollum, Simmonds, Becker and Shipley [1922] found that associated with the growth-promoting vitamin A in milk fat is another vitamin which is concerned with calcium deposition. Since milk is believed to contain only a

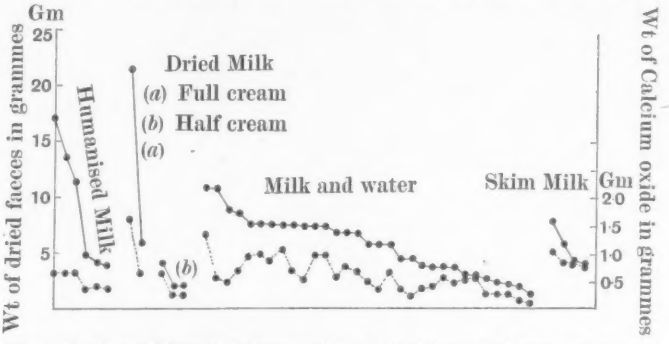

Fig. 2. To show relationship between weight of dried faeces and output of calcium oxide on different foods. ····· CaO ——— Faeces.

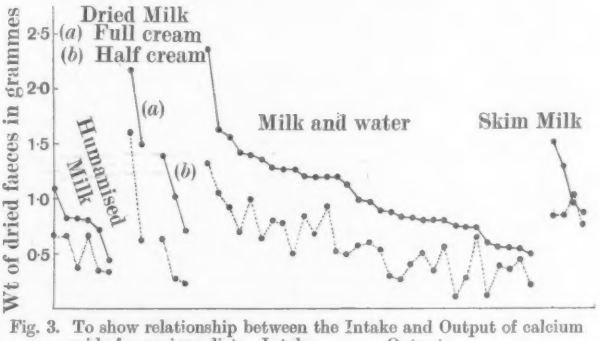

Fig. 3. To show relationship between the Intake and Output of calcium oxide for various diets. Intake ——— Output ·····

moderate amount of the calcium-depositing vitamin, and if it is distributed in common with the growth-promoting vitamin, either equally in the cream and the residue of the milk, or possibly in greater proportion in the cream than in the residue, the skim milk would contain only a very small amount—too small an amount in all probability to cause the deposition of calcium. If, however, this factor is stored like vitamin A in the body, the amount of calcium absorbed from the food on a reduced fat diet depends on the amount of the vitamin stored and the length of time during which the low fat diet has been taken. The case in this investigation showing a negative calcium balance had been on a low fat diet for seventeen days, long enough in all probability for the store of anti-rachitic (Mellanby) or calcium-depositing (McCollum, etc.) vitamin to have been entirely removed.

That the absorption of calcium depends on the fat intake as well as, or even more than, the calcium intake, is shown graphically in Fig. 4. For all the cases on ordinary milk and water mixtures, there is, generally speaking, a diminished calcium absorption with a diminished fat intake. This is also true for the dried milk mixture when the fat content is not too high. In the cases on humanised dried milk mixtures, although there is a high fat content,

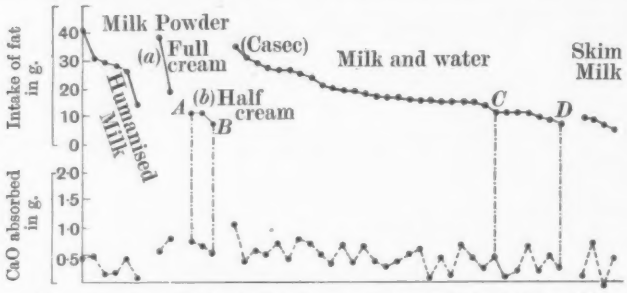

Fig. 4. To show the relationship between the weight of calcium oxide absorbed •-------• and the fat intake •——•

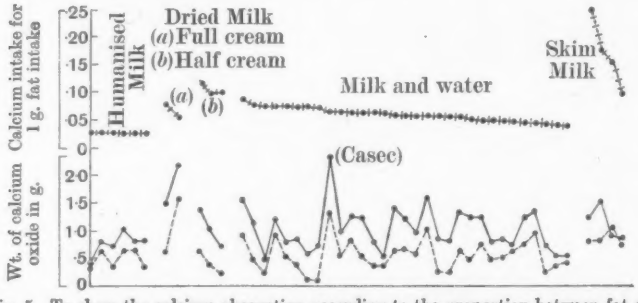

Fig. 5. To show the calcium absorption according to the proportion between fat and calcium intake. •—|—|—• calcium intake for 1 g. fat. •——• calcium intake in g. •-------• calcium output in g.

there is also a very low calcium intake, as shown in Fig. 3, compared with the other feeds, and consequently a low calcium absorption. This would indicate that an ample supply of calcium, together with an ample supply of fat results in a good absorption of calcium, and is in agreement with Holt's opinion previously stated. When the fat intake is less than 12 to 14 g. daily, the calcium absorption may be perilously low.

Fig. 5 indicates that there is also a certain relationship to be observed between the fat and the calcium intake to obtain the best absorption of calcium. The best absorption was obtained when there was from 0·04 to 0·078 g. of calcium oxide to 1 g. of fat in cow's milk and water mixtures, and the absorption was very good also when the intake of calcium oxide was as great as 0·12 g. to 1 g. of fat in the dried milk mixtures.

It is interesting to notice that the absorption of the calcium from the half-cream dried milk was slightly better than from cow's milk and water mixtures giving the same fat intake. (Compare *A*, *B* with *C*, *D* in Fig. 4.)

Observations on the absorption of fat from the half-cream dried milk mixtures show that it is very good also, the average for these same three cases being 91 %. Both these findings confirm the opinion previously formed from clinical observation that the half-cream dried milks often produce better results in infant feeding than the full-cream dried milks.

The difference in the amounts of calcium absorbed by the same infant on (*a*) a milk of fairly high fat content, and (*b*) a milk of low fat content after seventeen days on this diet, is shown as follows:

% fat in food	Calcium intake per day g.	% absorption
2·0	0·44	34·0
0·8	0·97	0

Since the absorption of calcium is very largely affected by the kind of milk (liquid, dried by the roller process, or humanised and dried by the spray process), the average percentage absorption of 43·0 % for 48 cases has little value. The greater number of cases, however, show an absorption between 30 and 60 %, those below being practically all cases on skim milk or a dried milk mixture with a high fat content. The latter may be explained perhaps on the ground of the large weight of faecal matter generally produced on these diets when the fat content of the milk mixtures is very high, and consequently the output of saponified fat comparatively large. The average weight of the dried faeces for the group of six cases on humanised dried milk with a fat content varying from 3 2 to 4·9 % was 9·1 g. in 24 hours, while the average weight of dried faeces for the group of 31 cases on cow's milk and water mixtures with a fat content varying between 1·4 and 3·3 was 5·8 g. in 24 hours. The average output of saponified fat for six of these cases on the humanised dried milk mixtures was 2·6 g. in 24 hours, and for 31 cases on milk and water it was 1·6 g. in the same period (see Table II).

Table II. *Saponified Fat.*

No. of cases	Food	Output of sap. fat g.	Possible CaO as soap g.	Total CaO output g.	Possible CaO as phosphate g.
6	Humanised milk	2·6	0·26	0·51	0·25
2	Dried milk full-cream	3·8	0·38	1·11	0·74
3	,, half-cream	0·6	0·06	0·38	0·32
1	Condensed	1·7	0·17	0·29	0·12
31	Milk and water	1·6	0·16	0·57	0·41
4	Skim milk	0·6	0·06	0·87	0·80

Absorption per Kilogram of Body-weight. While the actual weight of calcium oxide absorbed by these infants during observation varied between 0 and 1·70 g. in 24 hours, with an average of 0·44 g., the amount absorbed per kilogram of body-weight varied from 0 to 0·33 g., with an average of 0·12 g. Holt, Courtney and Fales state that the average absorption of calcium oxide by healthy infants on a diet of cow's milk is 0·09 g. per kilo.

Fig. 6 shows the relationship between the weight of calcium oxide and the fat absorbed per kilogram of body-weight. From this graph it can be seen that to ensure an absorption of 0·1 g. of calcium oxide, the fat must be between 2 and 6 g. per kilo. Holt states that the fat intake should be not less than 4 g. per kilogram to ensure an absorption of 0·09 g. of calcium oxide in healthy children on cow's milk. This graph shows quite clearly that with the high fat intake found with the humanised dried milk mixtures, the absorption of calcium is poor.

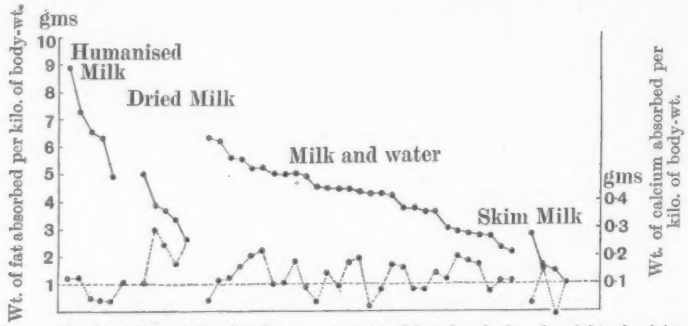

Fig. 6. To show the relationship between weight of fat absorbed and weight of calcium oxide absorbed per kilo. of body-weight. •——• fat. • - - - - - • calcium.

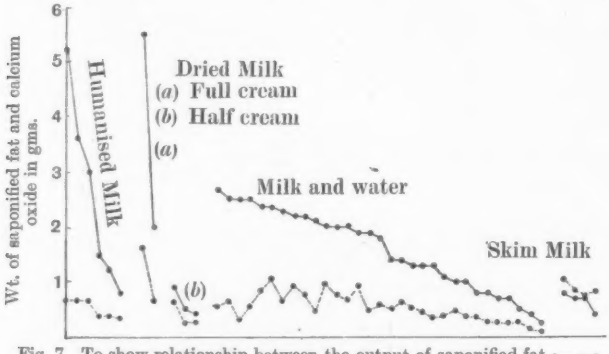

Fig. 7. To show relationship between the output of saponified fat •——• and calcium oxide • - - - - - • daily.

Since the average absorption of calcium oxide for the group of half-cream dried milk is 0·26 g. per kilo. of body-weight, it is evident that in some instances an amount of calcium much greater than what is thought to be the normal amount can be absorbed by atrophic children. Observations show also that the fat absorption of atrophic children is sometimes greater than the normal for healthy children [Parsons, 1924, 1]. Fourteen out of 31 cases on milk and water showed an absorption per kilo. of body-weight above the average of 0·12 g. With one exception these correspond to an intake of between 0·040 and 0·078 g. of calcium oxide for 1 g. of fat intake and the percentage of fat in the food was between 1·6 and 3·1.

The results obtained by Holt, Courtney and Fales for 30 cases of bottle-fed healthy children, from 2 to 16 months, some having cod-liver oil, and by Utheim for 17 cases of atrophic infants from 3 weeks to 15 months are given for comparison.

	Av. % absorption of calcium oxide		Av. absorption of calcium oxide		Av. daily excretion of calcium oxide	
	%	Range %	g.	Range	g.	Range
Holt *et al.*	37·2	0 to 66·7	0·081	−0·064 to 0·133	0·75	0·34 to 1·58
Utheim	24·9	0 to 68·5	0·067	−0·48 to 0·309	0·71	0·125 to 2·136
Hickmans	43·0	0 to 83·7	0·12	−0·02 to 0·33	0·60	0·09 to 1·60

(These figures include the 39 cases quoted by Parsons [1924, 2].)

The observations on the calcium metabolism of infants recorded before Holt's paper of 1920 show much lower absorption figures, due probably to the different methods used for the estimations, and are consequently not shown here for comparison.

The Relation between the Excretion of Calcium and Saponified Fat. Fig. 7 shows that with a general decrease in the output of saponified fat, there is also a similar though not parallel decrease in the calcium oxide output. It is important to notice that in the cases on a skim milk diet, the weight of the calcium oxide excreted is greater than the weight of the saponified fat (expressed as fatty acid). This would be equally true if the weight of all the saponified fat were expressed in terms of calcium soap. The excess therefore must be excreted as calcium phosphate or calcium carbonate. There was sometimes a slight effervescence when dilute acid was added to the dried faeces. No estimation of the phosphorus has yet been done on these specimens, but theoretical considerations indicate that the weight of calcium oxide possibly held as phosphate is greatest in the group on skim milk (see Table II).

Telfer [1922] states that 20 to 30 % of the total calcium oxide excreted normally exists in the form of calcium soap. For this group of 31 cases on milk and water the average figure for the amount of calcium oxide which could possibly be held as soap is within this range, but the proportion is considerably greater in the cases on humanised milk powder mixture with a high fat content (54 %), and less for the cases on skim milk (13 %).

Summary.

(1) Many atrophic infants can absorb calcium as well as normal infants, and in many cases can absorb larger amounts per kilo. of body-weight than is usually absorbed by normal children.

(2) In atrophy an ample intake of fat and calcium is beneficial.

(3) When the fat content is low, while the calcium content is high, this diet being continued for any length of time, the absorption of calcium may reach a perilously low level and there may be a negative balance.

(4) The calcium output depends somewhat on the calcium intake but more so on the fat intake. The fat intake should be not less than 12 to 14 g. daily.

(5) The best absorption takes place when there is a definite relationship between the fat and calcium intake, namely, when there is from 0·04 to 0·078 g. of calcium oxide for 1 g. of fat in milk and water mixtures; the absorption is also very good when the calcium oxide is as high as 0·12 g. in a certain dried milk mixture.

(6) The average percentage absorption of calcium oxide for 48 cases was found to be 43·0, but there is a great difference in the percentages shown with different diets.

(7) The actual weight of calcium oxide absorbed varied from 0 to 1·70, with an average of 0·43 g. in the 24 hours.

(8) The amount of calcium oxide absorbed per kilo. of body-weight varies from 0 to 0·33 g. with an average of 0·12 g.

(9) To ensure an absorption of 0·1 g. per kilo. of body-weight, the fat must be between 2 and 6 g. per kilo.

(10) Different foods have different calcium values. A variety of dried milk, made by the roller process shows a high amount of calcium absorbed. A dried humanised milk powder made by the spray process shows a very low value, while ordinary milk and water mixtures give an intermediate value.

REFERENCES.

Findlay, Paton and Sharpe (1921). *Quart. J. Med.* **14**, 352.
Holt, Courtney and Fales (1920). *Amer. J. Dis. Child.* **19**, 97.
Hopkins (1920). *Biochem. J.* **14**, 725.
Marriot (1920). *Amer. J. Dis. Child.* **20**, 461.
—— (1921). *Brit. J. Child. Dis.* **18**, 129.
McCollum, Simmonds, Becker and Shipley (1922). *J. Biol. Chem.* **53**, 293.
McCrudden (1920). *J. Biol. Chem.* **10**, 55.
Parsons (1924, 1). *Lancet*, i. 687.
—— (1924, 2). *Lancet*, i, 729.
Telfer (1922). *Quart. J. Med.* **16**, 45.
Utheim (1922). *J. Metab. Research*, **1**, 801.

CXXIV. THE PRODUCTION OF ORGANIC COMPOUNDS OF SULPHUR IN BACTERIAL CULTURES WITH SPECIAL REFERENCE TO GLUTATHIONE.

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There is an extensive literature which deals with the production of sulphides by bacteria. Without attempting a detailed review of this literature it will be sufficient to draw attention to some of the more important facts which have been established.

It seems to be clear that a number of bacteria are able to produce H_2S from media containing sulphur, sulphite or cystine; but that H_2S is rarely produced from taurine or sulphates. Further, the importance of cystine as a source of sulphide has been demonstrated. Sulphide is produced when bacteria are cultivated in synthetic media containing cystine but no other source of sulphur [Sasaki and Otsuka, 1912; Myers, 1920]. As sources of sulphide the different peptones appear to vary considerably and even from sample to sample, when used in culture media for the demonstration of sulphide production by bacteria. Certain results, however, have been constantly obtained with different media and by different observers; such are active sulphide production by *B. paratyphosus* B., *B. enteritidis*, *B. proteus*, *B. cloacae*, *B. typhosus* and absence of sulphide production by *B. paratyphosus* A and the dysentery bacilli [Myers, 1920; Thompson, 1921; Tilley, 1923]. The formation of volatile sulphides by anaerobes has been frequently noted [Med. Res. Council, 1919].

It is interesting that Thompson observes a general although not invariable rule that bacteria in the coliform group which ferment lactose do not form sulphide. Tanner notes that yeasts possess much greater power than bacteria for reducing both organic and inorganic compounds of sulphur.

The observations with regard to the formation of organic compounds of sulphur by bacteria are not numerous. Sasaki and Otsuka [1912], working with pure cultures and synthetic media, were unable to demonstrate mercaptan formation. However, Kondo [1923], working with synthetic media containing *l*-cystine, showed that *B. proteus* constantly and *B. coli* occasionally produced mercaptan provided one of the following were present in the medium glucose, lactose, saccharose, glycerol or histidine. No other amino acid tested could take the place of histidine. He also obtained presumptive evidence of the production of ethyl sulphide or some other alkyl sulphide. H_2S was invariably produced under these conditions.

In view of the rather scanty existing evidence for the production of organic compounds of sulphur by bacteria it was of particular interest that Hopkins in the account published in 1921 of his important discovery of the compound glutathione referred to its probable occurrence in bacterial cultures. These observations were limited to the occurrence of the nitroprusside reaction in cultures of such bacteria as he had tested. At all events no mention is made of the isolation of the compound from bacterial cultures.

It was considered probable by Hopkins [1921; Hopkins and Dixon, 1922] that traces of H_2O_2 were formed in the course of oxidations in which glutathione acted as an accelerator of respiration in the presence of oxygen.

As we were engaged about that time in an investigation of the production of H_2O_2 by bacteria [McLeod and Gordon, 1922], it seemed to us to be desirable to get more extended information with regard to the occurrence of substances reacting with nitroprusside in bacterial cultures. We hoped to establish some correlation between production by bacteria of H_2O_2 on the one hand and of substances reacting with nitroprusside on the other.

The method of carrying out the reaction was as follows: a test-tube was filled to the depth of half an inch with $(NH_4)_2SO_4$ crystals; then 10 cc. of broth culture, 0·5 cc. of a 5 % solution of nitroprusside and lastly 5–7 cc. of strong NH_4OH were added.

All the cultures of anaerobes tested gave strong reactions and in so far as could be judged with a method, which is not particularly well adapted to quantitative determinations, the reactions with the more strict anaerobes appeared to be the stronger.

In observations with cultures of bacteria which could grow aerobically tests were made both with and without previous reduction. Reduction was effected by an aluminium mercury couple.

The strongest positive reactions were obtained with cultures of *B. paratyphosus* B. and of the cholera vibrio, weaker positives were obtained with *B. gaertner* and other strains allied to *B. paratyphosus* B. and with some strains of *B. coli*, with *B. typhosus* and with *B. proteus*. Doubtful results were got with some strains of *Staphylococcus aureus*, negative with others. Reactions were invariably negative with *Gonococcus*, *Meningococcus*, *Pneumococcus* and strains of Influenza bacilli, *Streptococci* (haemolytic and non-haemolytic), *Staphylococcus albus*, Flexner, Y, and Shiga strains of dysentery bacilli and with *B. paratyphosus* A. The nitroprusside reactions obtained with cultures of anaerobes or with cultures of *B. paratyphosus* B. grown under anaerobic conditions were apparently a good deal stronger than those got on reducing the original broth for 5–10 minutes with an active HgAl couple with a view to reducing the oxidised glutathione from the meat extract. Some part of this reaction may be due to inorganic sulphides, mercaptans or acetone but inasmuch as the reaction is little or not at all decreased by prolonged boiling, such compounds can only account for a small fraction of the reacting substances.

It was found, however, that a heavy suspension of a surface growth of *B. welchii* on a solid medium, whether boiled or unboiled, fresh or reduced, never gave a definite nitroprusside reaction.

This and some experimental work in which old and recent yeast extracts were compared with bacterial cultures in regard to rates of disappearance of the nitroprusside reaction led us to wonder whether the nitroprusside reaction occurring in bacterial cultures was not entirely due to a very effective reduction of the oxidised glutathione originally present in the medium.

The accuracy of this supposition was tested in two ways; the first was an investigation of the power of bacteria to produce a nitroprusside reaction in broth freed from substances reacting with nitroprusside. This substance, presumably glutathione, can be destroyed by heating with 1–2 parts per 1000 of H_2O_2. It was not often possible however to destroy the residual H_2O_2 completely by subsequent boiling of the broth. R. Wolffenstein [1894] draws attention to the fact that the liability of H_2O_2 to destruction by heating has been exaggerated and shows that in the absence of (*a*) traces of heavy metals, (*b*) substances of alkaline reaction, and (*c*) solid particles of any kind, it is relatively heat stable. We therefore had recourse to three different methods of getting rid of the residual H_2O_2. These were:

(1) Shaking with MnO_2 powder, the MnO_2 being subsequently removed by filtration.

(2) Warming with 0·5–1 % of glucose in the presence of a trace of $FeSO_4$.

(3) Shaking with a heavy emulsion of staphylococci rich in catalase [McLeod and Gordon, 1923] and filtering off the staphylococci by means of a porcelain candle (Maassen type).

The broth resulting from each of those three forms of treatment was proved to be free from glutathione as it gave no nitroprusside reaction after prolonged reduction, $\frac{3}{4}$–1 hour, with an active couple.

Both *B. welchii* and *B. paratyphosus* B. were successfully cultivated in all three media although the growths were distinctly less copious than in the original broths. Such cultures always failed to give any trace of nitroprusside reaction. Cultures of these bacteria in the same broth subjected to the same treatment with staphylococci but not oxidised by H_2O_2 gave frank nitroprusside reactions.

The second way of testing our theory was to observe whether by means of prolonged reductions by aluminium mercury couples it was possible to produce reactions in ordinary meat extract broth similar in intensity to those observed in bacterial cultures. The reductions to which reference is made in the earlier part of the paper were not carried out for more than 10–15 minutes. It was found that broth reduced for 1 hour by an active couple gave a reaction little if at all inferior to that obtained in the same broth after the growth of an anaerobe. We have therefore been led to conclude that bacteria do not produce glutathione when cultivated in nutrient broth and that the nitroprusside reactions observed in bacterial cultures are mainly or entirely due to the

special aptitude of the bacteria concerned for reducing the residual oxidised glutathione present in the medium. The nitroprusside reaction in a bacterial culture in meat extract broth is therefore an indication of the reducing powers of that bacterium and very possibly an indication of its capacity to utilise glutathione in carrying out some of the oxidations required for its metabolism.

Glutathione would not appear to play any part in the production of H_2O_2 by bacteria of the streptococcal group although it may in the case of the anaerobes. The last subject we hope to deal with in a subsequent communication. A point of some importance arising from these observations is the marked permanence of glutathione in the oxidised condition in media prepared from meat extract and the possibility that it is one of the important constituents of meat extract for promoting the growth of certain bacteria.

Summary.

(1) A distinct nitroprusside reaction has been observed in 24–48 hour cultures of the following bacteria—all anaerobes tested (six, including *B. welchii*, *B. tetani* and *Vibrion septique*), *B. paratyphosus* B. and most allied species, *Cholera vibrio* (one strain), *B. typhosus*, *B. pyocyaneus*, some strains of *B. coli* and *B. proteus*.

(2) No distinct nitroprusside reactions have been observed in cultures of *Staphylococcus*, *Streptococcus*, *Pneumococcus*, *Gonococcus*, *Meningococcus*, *B. influenzae*, *B. Morax-Axenfeld*, *B. paratyphosus* A. or of the dysentery bacilli (Flexner, Y, and Shiga types).

(3) Where a facultative anaerobe gives a nitroprusside reaction it is much stronger if the bacterium is grown in anaerobic or nearly anaerobic conditions.

(4) The thermo-stable substance reacting with nitroprusside detected in bacterial cultures is not produced by the bacteria but is the oxidised glutathione or some related compound originally present in the medium and reduced by the bacteria in the course of their growth.

(5) Bacteria therefore do not produce glutathione or substances allied to it reacting with nitroprusside.

(6) Bacteria capable of reducing glutathione probably utilise it when growing under anaerobic conditions to accelerate the oxidations necessary to their metabolism.

We have pleasure in acknowledging our indebtedness to the Medical Research Council for a grant in aid of this work.

References.

Hopkins (1921). *Biochem. J.* **15**, 286.
Hopkins and Dixon (1922). *J. Biol. Chem.* **54**, 527.
Kondo (1923). *Biochem. Z.* **136**, 198.
McLeod and Gordon (1922). *Biochem. J.* **16**, 499.
—— —— (1923). *J. Path. Bact.* **26**, 326.
Medical Research Council (1919), *Special Report Series*, **39**, 63.
Myers (1920). *J. Bact.* **5**, 231.
Sasaki and Otsuka (1912). *Biochem. Z.* **39**, 208.
Tanner (1918). *J. Amer. Chem. Soc.* **40**, 663.
Thompson (1921). *J. Med. Research*, **42**, 383.
Tilley (1923). *J. Bact.* **8**, 115, 287.
Wolffenstein (1894). *Ber. deutsch. chem. Ges.* **77**, 3307.

CXXV. STUDIES ON SELECTIVE BACTERICIDAL ACTION.

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Cases of selective bactericidal action have been frequently observed by different investigators.

In the course of a comprehensive research on the germicidal power of nitroso-compounds, the authors have, however, noticed a very uncommon type of selective action. From the results obtained it appears that *Bacillus pyocyaneus* is abnormally sensitive to the action of the so-called physico-chemical class of germicides, *e.g.* phenol, alcohol, whilst it is very resistant to the action of germicides that owe their efficacy to definite chemical action upon the protoplasmic constituents of the organism, *e.g.* nitroso-compounds, salts of heavy metals, etc. As the results may contribute to our knowledge of the chemical constitution and colloidal structure of micro-organisms, and further work on these lines would seem to offer a promising field for research, the main results of the investigation are summarised and discussed in the present paper.

The method employed for estimating germicidal power may be briefly described as follows.

A culture of the particular micro-organism used was incubated for a definite period in the presence of various concentrations of the germicides under examination, and the effect on the growth of the organism was observed. The exact technique consisted in first adding to definite volumes of lemco-peptone broth (8 cc.) varying quantities of sterile distilled water, in such amount that on subsequently adding the desired volume of disinfectant solution, the total volume would be 10 cc. Before the addition of the germicide, however, two drops of a 24 hours' culture of the organism were added to each tube. After well shaking, the disinfectant was then introduced. After further mixing the tubes were incubated at 37° for two days, at the end of which the tubes were examined. In this way it was possible to determine the exact concentration which was just sufficient to prevent the growth of the organism. When several compounds are being compared by this method, it is necessary to test them simultaneously, to exclude errors due to variations in the organisms. Although it would appear that inhibitory action is being determined by this method, yet there is evidence that we are in reality measuring germi-

cidal power, since by sub-culturing blank tubes into fresh broth, no growths occurred in the sub-culture tubes. Different varieties of the organisms were used in order to exclude possible errors due to idiosyncrasy of any one culture. It is of importance to point out that the selective action of the physico-chemical group of germicides upon *B. pyocyaneus* is not necessarily shown in short period experiments, but the 48 hours' test used in this investigation has been found to be fully adequate for the purpose of differentiation.

The marked germicidal action of *p*-nitrosophenol and *p*-nitrosoaniline was first pointed out by Cooper and Woodhouse [1923], and in the present communication a large number of nitroso-compounds—some of quite a different type—have been examined.

The bactericidal powers of these substances are tabulated in Table I, and from a consideration of the results, the following conclusions may be drawn.

Table I. *Germicidal Concentrations of Nitroso-compounds.*

Substance	*B. coli communis**	*B. typhosus*	*B. pyocyaneus*
Phenol	1 in 550	1 in 680	1 in 870
p-Nitrosophenol	1 „ 18,000	—	1 „ 2,000
p-Nitroso-*o*-cresol	1 „ 16,500	—	1 „ 1,200
p-Nitroso-*m*-cresol	1 „ 16,500	—	—
p-Nitrosoresorcinol	1 „ 2,100	—	—
Dinitrosoresorcinol	1 „ 2,100	—	—
α-Nitroso-*β*-naphthol	1 „ 45,000	—	—
p-Nitrosoaniline	1 „ 120,000	1 in 200,000	1 in 33,000
p-Nitroso-methyl-aniline	1 „ 140,000	1 „ 270,000	1 „ 11,000
p-Nitroso-ethyl-aniline	1 „ 70,000	1 „ 160,000	1 „ 3,500
p-Nitroso-di-methyl-aniline	1 „ 170,000	1 „ 300,000	1 „ 8,500
p-Nitroso-di-ethyl-aniline	1 „ 50,000	1 „ 125,000	1 „ 1,400
p-Nitroso-*o*-toluidine	1 „ 200,000	—	1 „ 18,000
p-Nitroso-phenyl-methyl-nitrosamine	1 „ 18,000	1 „ 62,000	>1 „ 3,000
p-Nitroso-phenyl-ethyl-nitrosamine	1 „ 13,000	1 „ 140,000	>1 „ 3,000

* The culture used was Houston II. Results of much the same order were obtained with Escherich.

All the nitroso-derivatives tested are active germicides, nitrosoaniline and its homologues being more efficacious than the nitrosophenols, and the dinitroso-compounds.

The nitrosophenols are remarkable insomuch as the introduction of a methyl group results in a diminution in germicidal power, whilst in the case of the phenols it is well known that the cresols are much superior to phenol itself.

Nitroso- and dinitroso-resorcinol are less active than the monohydric nitrosophenols, while *α*-nitroso-*β*-naphthol is the most powerful of all the phenolic derivatives examined.

The homologous series of nitrosoanilines behave in a very peculiar manner. With the coliform organisms, the introduction of *methyl* groups slightly increases bactericidal power, whilst that of *ethyl* groups brings about an appreciable fall in efficacy. Nitroso-di-methyl-aniline is therefore a more active germicide than the isomeric nitroso-ethyl-aniline. Symmetrical substitution is thus more favourable than the dissymmetrical type, as has also been observed in the case of tellurium compounds [Morgan, Cooper and Burtt, 1924].

The results with *B. pyocyaneus* are different from those observed with *B. coli* and *B. typhosus*.

B. pyocyaneus is consistently more resistant than the latter organisms to the action of all the nitroso-compounds of the different types examined, while, on the other hand, it shows a specific susceptibility to the germicidal action of phenol itself. In both the nitroso-phenol and aniline series the introduction of alkyl groups also causes a marked diminution in bactericidal power in the case of *B. pyocyaneus*.

It is interesting, furthermore, to note that the substitution of a nitroso group in the secondary amine radicles of nitroso-methyl- and nitroso-ethyl-aniline, forming nitroso-nitrosamines leads to a collapse in germicidal action.

The anomalous behaviour of *B. pyocyaneus* referred to above suggests that this organism is abnormally susceptible to a germicide of the physico-chemical or colloid-precipitating type, *e.g.* phenol, whilst towards typical chemical germicides, such as nitroso-compounds, which readily condense with nitrogenous substances, it is extremely resistant. It was evident, however, that before any general conclusion could be reached, it would be necessary to test a large number of compounds of widely different chemical nature, and this work was therefore next undertaken and the results are tabulated in Table II.

A consideration of the above results shows that the compounds which are known to react chemically with the constituents of protoplasm and may therefor be classified as chemical germicides, e.g. nitroso-compounds, aldehydes, benzoquinone, hydroxylamine, salts of heavy metals (mercury and cadmium) are with one exception much more efficacious against *B. coli* than against *B. pyocyaneus*. The only exception is formaldehyde (and occasionally hydroxylamine), which attacks the two organisms almost with equal readiness. The tellurium-β-diketone which on account of its activity in extreme dilution is in all probability a chemical germicide, also exerts a selective action on *B. coli*. The results obtained with cadmium sulphate are of interest as the germicidal power of the salts of this metal has not apparently been examined previously. It is seen that cadmium sulphate is quite efficacious as a germicide, but is less active than mercuric chloride.

The results with the physico-chemical germicides are more complex, but quite significant, when the interfering factors are considered. It is seen in the first place that the homologous series of aliphatic alcohols, also benzyl alcohol, phenol, chloro-phenols, quinol, and toluquinol, differ from the above chemical germicides in preferentially attacking *B. pyocyaneus*. Thus, quinol is nearly four times as efficacious against *B. pyocyaneus* as against *B. coli*. These results show quite clearly that *B. pyocyaneus* is abnormally susceptible to the action of the physico-chemical group of germicides.

There are, however, some anomalous results that call for explanation. It has already been shown that the introduction of an alkyl group into the benzene

nucleus or amino-group of nitroso-compounds has the unusual effect of greatly diminishing the bactericidal action of nitroso-compounds against *B. pyocyaneus*. In the case of the aliphatic alcohols, this, however, does not hold, and the homologous series behaves quite consistently with both organisms. In ascending the series of phenols, however, whilst in the case of *B. coli*

Table II. *Differentiation between* B. pyocyaneus *and* B. coli.

	Bactericidal concentrations	
The alcohols	*B. pyocyaneus*	*B. coli* (Houston II)
Methyl alcohol	1 in 14	1 in 9
Ethyl alcohol	1 „ 43	1 „ 21
Propyl alcohol	1 „ 67	1 „ 43
Isopropyl alcohol	1 „ 43	1 „ 30
Benzyl alcohol	1 „ 260	1 „ 177
Monochlorohydrin	1 „ 60	1 „ 60
The phenols		
Phenol (i)	1 „ 920	1 „ 650
(ii)	1 „ 780	1 „ 540
o-Cresol	1 „ 1,215	1 „ 1,215
m-Cresol	1 „ 1,215	1 „ 1,215
p-Cresol	1 „ 1,215	1 „ 1,215
Thymol (i)	1 „ 1,255	1 „ 4,700
(ii)	1 „ 835	1 „ 4,300
o-Chlorophenol	1 „ 4,850	1 „ 2,430
p-Chlorophenol (i)	1 „ 4,160	1 „ 4,160
(ii)	1 „ 4,160	1 „ 3,500
Picric acid	1 „ 290	1 „ 600
The quinols		
Quinol	1 „ 2,550	1 „ 710
Toluquinol	1 „ 1,040	1 „ 700
p-Xyloquinol	1 „ 1,240	1 „ 1,740
Thymoquinol	1 „ 330	1 „ 1,100
The aldehydes		
Formaldehyde (i)	1 „ 17,300	1 „ 17,300
(ii)	1 „ 16,300	1 „ 16,300
(iii)	1 „ 16,300	1 „ 16,300
Glyoxal	1 „ 466	1 „ 900
The amines		
Aniline (i)	1 „ 355	1 „ 250
(ii)	1 „ 333	1 „ 225
Benzylamine	1 „ 1,700	1 „ 807
Phenylethylamine	1 „ 1,340	1 „ 1,340
Hydroxylamine hydrochloride (i)	1 „ 11,000	1 „ 42,000
(ii)	1 „ 10,000	1 „ 10,000
The quinones		
Benzoquinone	1 „ 6,000	1 „ 10,000
Inorganic compounds		
$HgCl_2$ (i)	1 „ 200,000	1 „ 450,000
(ii) less than	1 „ 50,000	1 „ 410,000
(iii)	1 „ 33,300	1 „ 200,000
$CdSO_4$ less than	1 „ 8,400	1 „ 98,000
NaCl (i)	1 „ 15·4	1 „ 15·4
(ii)	1 „ 19·0	1 „ 15·0
$(NH_4)_2SO_4$ (i)	1 „ 7·7	1 „ 7·7
(ii)	1 „ 9·5	1 „ 9·5
Tellurium compounds [Morgan, Cooper and Burtt, 1924].		
Tellurium dipropionyl-methane	1 in 250,000	1 in 9,000,000

germicidal action increases to a marked degree, with *B. pyocyaneus* there is only a very slight increase. The difference is so significant that thymol, which is eight times as active as phenol against *B. coli*, is only very slightly more efficacious than phenol with *B. pyocyaneus*. Exactly analogous results have been obtained with the alkyl derivatives of aniline.

In ascending the series of the homologous quinols, moreover, with *B. coli*, there is only a moderate increase in bactericidal power, while with *B. pyocyaneus*, there is actually a very marked diminution in efficacy. Consequently, thymoquinol, as in the case of thymol, is approximately four times as active against *B. coli* as against *B. pyocyaneus*.

There is no doubt that alkyl substitution in aromatic compounds has a very anomalous effect on germicidal power when the latter bacillus is used as the test organism. As a consequence, thymol and thymoquinol behave exceptionally in attacking *B. coli* more rapidly than *B. pyocyaneus*, and their specific action as physico-chemical germicides is thus obscured.

It is noteworthy that the three cresols have a higher germicidal power than the isomeric benzyl alcohol.

Sodium chloride, ammonium sulphate, and monochlorohydrin which may also be regarded as physico-chemical germicides, although they do not exhibit the preferential action of the above alcohols and phenols, yet differ from the majority of chemical germicides in exerting approximately an equal effect on the two organisms. In this respect they approach the exceptional case of formaldehyde referred to above.

It is interesting to observe that the amines examined, e.g. aniline and benzylamine, appear to belong to the class of physico-chemical germicides, as they exert a selective action upon *B. pyocyaneus*, in this respect resembling the analogous hydroxy compounds, phenol and benzyl alcohol.

Picric acid, however, is a more active germicide against *B. coli* than against *B. pyocyaneus*, and thus differs from the alcohol and phenol group of disinfectants.

Several investigators have drawn an analogy between the action of heat and alcohol upon proteins; Lepeschkin [1923] in fact has shown that the addition of alcohol increases the velocity of heat-denaturation of proteins. We therefore anticipated that *B. pyocyaneus*, being selectively attacked by alcohol and phenol, would be more susceptible to the action of hot water than *B. coli*. Filtered cultures (24 hours in broth) of *B. coli* and *B. pyocyaneus* were therefore heated in the same thermostat at 56° and sub-cultures made into fresh broth at regular intervals. It was found that, whilst a period of 75 minutes was required for the complete destruction of *B. coli*, *B. pyocyaneus* was killed off in the much shorter period of 15 minutes.

Quantitative experiments involving counting on agar plates were next carried out. Diluted filtered cultures of the two organisms were heated at 56°, each culture being provided with a stirrer in order to diminish the errors due to sampling, and sub-cultures were made at convenient intervals into

agar-plates. The latter were incubated at 37° for 48 hours, and the colonies then counted.

The results were as follows:

Table III.

Time at 56°, mins.	*B. coli*, Nos. per drop	Time, mins.	*B. pyocyaneus*, Nos. per drop
0	28,200	0	24,000
2	4,920	2	7,000
4	3,450	5	200
7	720	6	30
12	45	8	25
14	35	11	3
18	18		
22	2		
27	1		
32	1		

The above observations thus confirm the preliminary tests. It is evident that *B. pyocyaneus* is considerably more susceptible to the action of heat than *B. coli*, and the results as a whole strongly suggest a very close similarity in the mechanism of disinfection by heat and alcohol or phenol.

Summary.

1. Several compounds, e.g. nitroso-compounds, quinone, aldehydes, salts of heavy metals, hydroxylamine hydrochloride, which react with the constituents of protoplasm and can therefore be classified as chemical germicides, exert a more powerful bactericidal action upon *B. coli* than on *B. pyocyaneus*. Formaldehyde behaves exceptionally in attacking both organisms with equal readiness.

2. The aliphatic alcohols, benzyl alcohol, phenol, chlorophenols, quinol, and toluquinol differ from the foregoing compounds in attacking *B. pyocyaneus* more readily than *B. coli*. In the case of quinol, the selective germicidal action is so marked that this substance is four times as active against *B. pyocyaneus* as against *B. coli*.

3. The above observations show that, whilst *B. pyocyaneus* is very resistant to the action of *chemical* disinfectants, it is selectively attacked by the germicides of the so-called physico-chemical type, e.g. alcohol and phenol, the bactericidal action of which is associated with some specific effect upon the colloidal condition of the bacterial protoplasm.

4. *B. pyocyaneus* is also considerably more suceptible to the action of heat than is *B. coli*. The latter observation supports the view that there is a fundamental similarity in the germicidal action of heat and of phenol or alcohol.

5. The results as a whole afford evidence of a very uncommon case of selective bactericidal action, which differentiates between the chemical and physico-chemical classes of germicides, and also suggest that the colloidal

structure of *B. pyocyaneus* is characteristically susceptible to the destructive effect of heat and denaturating substances.

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REFERENCES.

Cooper and Woodhouse (1923). *Biochem. J.* **17**, 600.
Lepeschkin (1923). *Kolloid Z.* **32**, 100.
Morgan, Cooper and Burtt (1924). *Biochem. J.* **18**, 190.

CXXVI. THE ACTION OF PARALDEHYDE UPON PROTEINS AND LIPINS.

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The action of chloroform and other anaesthetics upon proteins and lipins has been extensively studied by several investigators, with the object of throwing light on the nature and mechanism of anaesthesia. No such work, however, seems to have been carried out up to the present in the case of paraldehyde to account for its characteristic pharmacological action. The experiments described in this paper were therefore planned with this object in view, and for the further purpose of ascertaining whether paraldehyde would be useful as a solvent or precipitant in the fractionation and purification of proteins and lipins.

Action upon Proteins.

In the first place, amino acids, and various proteins, e.g. gelatin, egg-albumin, caseinogen, were found to be insoluble in cold or warm paraldehyde. Gliadin was precipitated from alcoholic solution on the addition of paraldehyde, and egg-albumin and peptone were similarly precipitated from their solutions in molten anhydrous phenol.

Saturated aqueous solutions of paraldehyde, however, exerted no precipitating action upon colloidal solutions of gelatin, egg-albumin, serum-albumin, or starch.

Nucleic acid was quite insoluble in paraldehyde and a colloidal solution obtained by acidifying the slightly alkaline solution with dilute acetic acid was also unaffected by the addition of paraldehyde.

Action upon Cholesterol, Fatty Acids, and other Substances.

Paraldehyde freely dissolved cholesterol, higher fatty acids (palmitic and stearic), and wax (spermaceti) in the cold or on warming to 40°. On the addition of water or on merely cooling the warm solutions, the above substances crystallised out. Paraldehyde was also readily miscible with fats. Sodium cholate, however, was insoluble in paraldehyde, and was precipitated from alcoholic solution by the addition of this substance. Aqueous solutions were unaffected, but permanently emulsified the paraldehyde.

Action on Lipins.

Ordinary commercial lecithin was immediately precipitated from ethereal solution by paraldehyde, and this was still found to be the case after purification of the lecithin by precipitation from ethereal solution with acetone.

Alcoholic solutions of lecithin were, however, unaffected, but an aqueous colloidal solution was readily precipitated by the addition of a saturated watery solution of paraldehyde either at ordinary or body temperature.

Experiments were next carried out to ascertain in what way paraldehyde would behave in the fractionation of the constituents of nerve tissue.

A sheep's brain was well minced and repeatedly extracted with absolute alcohol at 40°. The extract was filtered and cooled at 0°. The precipitate which gradually formed was collected and washed with cold alcohol. This substance (the so-called "protagon") was found to be quite insoluble in cold or warm paraldehyde. A portion of it was extracted with ether. The ether-insoluble fraction, consisting of cerebrosides, was also insoluble in paraldehyde, and the cerebrosides were moreover precipitated by paraldehyde from their solutions in pyridine and hot alcohol.

The ether-extract from the original protagon was next treated with paraldehyde, and the phosphatides were found to be precipitated.

On account of the cyclic ether structure of paraldehyde, it was of interest to compare its precipitating action with that of ether itself, and also with that of acetone. The results are compared in the following table:

	Paraldehyde	Ether	Acetone
Proteins			
(1) Aqueous colloidal solutions	No action	Precipitation	Precipitation
(2) Solutions in anhydrous solvents	Precipitation	,,	,,
Phosphatides			
(1) Aqueous colloidal solutions	Precipitation	No action	Precipitation
(2) Ethereal solutions	,,	,,	,,
Cerebrosides	Insoluble	Insoluble	Insoluble
Cholesterol, fat, wax, fatty acids	Soluble	Soluble	Soluble

It is seen that paraldehyde differs from ether in readily precipitating phosphatides and in having no action on aqueous colloidal solutions of proteins. From acetone, however, it only differs in the latter respect.

From these observations it would appear that the pharmacological action of paraldehyde is associated with its physico-chemical action upon the colloidal suspensions of the lipins in the nerve tissue. It would also appear that paraldehyde may prove useful as a fractionating medium in the isolation and purification of the various constituent lipins of nerve or other tissues.

Summary.

1. Paraldehyde readily precipitates proteins from anhydrous solvents, but has no action on their aqueous colloidal suspensions or on those of nucleic acid.

2. Paraldehyde precipitates lecithin from both water and ether. It also precipitates cerebrosides from their solutions in pyridine and hot alcohol.

3. Paraldehyde is a solvent for cholesterol, wax, fats and the higher fatty acids.

4. Paraldehyde may thus prove to be of service in the fractionation and purification of the lipins of nerve or other tissues.

5. The pharmacological action of paraldehyde would seem to be associated with its observed physico-chemical action on the colloidal suspensions of the lipins of nerve tissue.

CXXVII. METHOD OF ESTIMATING MINUTE QUANTITIES OF IODINE IN BIOLOGICAL MATERIAL.

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The importance of iodine to the animal organism is becoming increasingly apparent, and at the outset of an investigation into this question we found it necessary to review the existing methods for estimating minute quantities of iodine in organic substances, primarily with regard to thyroid gland studies and also with a view to ascertaining if possible the iodine content of pastures and other foodstuffs.

A number of methods have been described for such estimations, and a survey of the literature on the subject reveals many figures for foodstuffs and like material which appear to us to need some investigation. For 100 g. of dried material some methods claim an accuracy of 0·03 mg. %, others 0·005 mg. % and figures even much smaller than these are quoted.

A short discussion of each of the methods investigated by us will be made, finishing with a description of the Kendall method which in its broad outlines we have adopted.

Hunter's method [1910]. The organic matter is fused with a mixture consisting of potassium carbonate, sodium carbonate and potassium nitrate. The sodium iodide thus fixed is converted to iodic acid by oxidation with sodium hypochlorite, and, after boiling off the excess of chlorine, the iodine is liberated from the iodic acid by means of an excess of potassium iodide, according to the equation

$$5KI + HIO_3 + 5HCl = 5KCl + 3H_2O + 3I_2$$

and titrated with a solution of approximately 0·005 *N* sodium thiosulphate. The outstanding advantage of this method is the titration of six times the amount of "original" iodine.

In analysing samples of seaweed, however, we found that considerable loss of iodine took place during the fusion process and further, owing to the formation of oxy-chlorine compounds [Kendall, 1912], which subsequently interfered with the titration, the results obtained were never reliable.

Hanzlik's method [1910]. This method consists in (*a*) desiccation in sodium hydroxide, (*b*) fusion and oxidation with sodium nitrate and sodium carbonate, (*c*) acidification of the extracted melt with sulphuric acid and the extraction

of the free iodine with chloroform. The chloroform is separated from the rest of the solution, washed, and the iodine titrated direct with $N/10$ sodium thiosulphate until the chloroform becomes colourless.

This method has been used by us with some success where the quantity of iodine present is fairly large, e.g. 0·1 g., but for amounts less than this we find it difficult to achieve complete extraction of the iodine by the chloroform.

During neutralisation with sulphuric acid free iodine is very liable to escape, and to obviate this risk we found it advisable, by means of a simple contrivance, to pass the gases evolved during neutralisation through chloroform which was subsequently used for extracting the rest of the iodine present.

The washing of the chloroform completely free from acid was also found difficult, and considerable loss of iodine may occur here.

When minute quantities of iodine are present, it is almost impossible to see the iodine colour in the chloroform and the titration becomes increasingly difficult.

Krauss' method [1915]. This is a colorimetric method based on the fact that palladous chloride precipitates iodine from dilute solutions as palladous iodide. This gives a deep brown coloration which can be compared colorimetrically with standards of known strength. We were unable to get satisfactory results with this method, largely on account of the difficulties experienced in completely extracting the iodine with the acetone-alcohol mixture and in differentiating between the colorations given when minute quantities of iodine were present.

Some of the earlier methods used are reviewed by Cameron [1914], notably those of Baumann, and Bourcet. The latter employed large quantities of material, and his method seems impracticable for general application where rapidity and convenience combined with accuracy are aimed at.

Kendall's method [1912, 1914, 1920]. The method which (with slight modifications) was found by us to be the most satisfactory is that of Kendall, and our results place its absolute limit of accuracy at 0·0001 g. %. The maximum amount of material which can conveniently be used is 5 g. The minimum amount of $N/200$ sodium thiosulphate with which a change of colour can be observed is 0·05 cc. which is equivalent to 0·0000317 g. iodine (i.e. 0·0000053 g. of "original" iodine). Should an excess of one drop be used in the titration, which is within the reasonable limits of experimental error, then it is obvious that the error in the percentage is materially increased, if such a minute quantity is present in the material to be analysed. The colour given by starch when such small quantities of iodine are present is also exceedingly faint, and we feel that figures quoted even below 0·0002 % are unreliable.

As has been stated, many workers claim by their methods an accuracy far more delicate than this, and one finds in the literature figures for foodstuffs with as low an iodine content as 0·0000005 % [Fellenberg, 1923].

We are in complete agreement with the findings of Bohn [1916], who, it appears, experienced the same difficulties as ourselves and whose results are

tabulated like those of Forbes [1916], viz. in a qualitative rather than a quantitative manner.

We have adopted the Kendall method in its broad outlines, but would make the following suggestions.

(1) We found it unnecessary to control the acidity during the boiling with bromine. Our experience has been that when neutralising the dissolved fusion melt, an excess of 3 cc. of syrupy phosphoric acid gives the necessary acidity for boiling off the nitrous acid. No further addition of phosphoric acid is needed before final titration. (2) In the initial fusion of pastures a considerable quantity of potassium nitrate is required completely to oxidise the large amount of carbonaceous matter (5 g. samples taken).

On acidification with phosphoric acid, nitrous acid is evolved in fair quantity owing to the reduction of the nitrate during fusion. The final titration would be impossible were this not boiled off, and an excess of 3 cc. of phosphoric acid is found sufficient for this purpose.

The pink colour of the just acid solution (methyl orange as indicator) will persist for a few moments, but is quickly destroyed by the liberation of the nitrous acid on the addition of 3 cc. excess of phosphoric acid.

The solution is now boiled for 15–20 minutes and disappearance of the last traces of nitrous acid may be watched by means of potassium iodide starch paper. We find that 2 cc. of sodium bisulphite added at this point is sufficient to reduce any oxidising agents which would interfere with the iodide-iodate reaction, and the solution is reboiled for 10 minutes or so to expel the excess of sulphur dioxide.

(3) To prevent bumping during boiling, small pieces of unglazed porcelain, obtained from broken triangles were found by us to be as convenient and satisfactory an agent as any. This should be removed before the titration is carried out and a long glass rod with a flattened end serves for this purpose.

(4) The method adopted by us for adding the potassium iodide is to dissolve a small crystal (0·25 g.) in about 20 cc. of water in a small beaker and add this immediately to the solution to be titrated. This is more satisfactory than adding a crystal direct as advised by Kendall, which causes a pink coloration with starch instead of a blue.

(5) In certain cases, notably pastures, owing to the large amount of sodium hydroxide used in the fusion process and the quantity of acid needed to neutralise this, the resulting solution becomes rather concentrated. This factor to some extent controls the speed of the iodide-iodate reaction which is sometimes slow. Five minutes are adequate to ensure the complete liberation of the iodine before the end point is finally determined. Forbes [1916] recommends the use of a little sodium chloride to accelerate the reaction and no doubt this is an advantage. The solution should remain clear for at least half an hour after titration, no blue colour reappearing.

Kendall's method as used by us is as follows:

Outline of Method.

(1) Fusion with sodium hydroxide and destruction of organic matter by potassium nitrate, with fixation of iodine as sodium iodide.

(2) Oxidation of iodine to iodic acid by bromine

$$I_2 + 6H_2O + 5Br_2 = 10HBr + 2HIO_3.$$

(3) Liberation of iodine from iodic acid by potassium iodide, and titration with sodium thiosulphate, using starch as indicator.

Reagents Required.

Sodium thiosulphate $N/200$ solution	Salicylic acid (pure)
Potassium bi-iodate standard solution	Potassium iodide crystals
Sodium bisulphite 20 % solution	Phosphoric acid (sp. gr. 1·75)
Soluble starch 1 % solution	Bromine

Details of Method.

Fusion. In the case of thyroid glands about 0·5 g. of fresh material and for pastures 5 g. previously dried and ground are taken.

The sample is accurately weighed into a nickel crucible of 5·9 c. in diameter, covered with a convenient amount of 40 % sodium hydroxide solution (10 cc. for thyroids, 35 cc. for pastures) and heated in an oven for about half an hour.

The nickel crucible is then placed in another of slightly larger capacity half filled with sand, and the whole is placed in a crucible furnace and heated gently over a moderate flame.

It will be found that the substance saponifies and froths considerably, and gentle stirring with a nickel rod is necessary to keep the melt from creeping over the sides of the crucible. In about 10 minutes the frothing ceases, most of the carbonaceous matter becomes burned off and the melt settles to the bottom.

At this point about 5 g. of solid sodium hydroxide (powdered or in flake) are added and the melt becomes liquid and more easily worked. Small quantities of potassium nitrate are added repeatedly until all the carbon is completely oxidised and further additions cause no bubbles or frothing. The crucible is removed from the sand-bath and allowed to cool. When cool it is placed in a beaker of 500 cc. capacity and the melt extracted with boiling distilled water.

In some cases a certain amount of insoluble residue is inevitable and the solution, which should amount to about 400 cc., is transferred to a conical flask of 700 cc. capacity, filtering if necessary. The cooled solution is neutralised with syrupy phosphoric acid, run in carefully from a burette using methyl orange as an indicator. From 2–3 cc. excess of acid should be used.

The solution is boiled for about 15–20 minutes to get rid of the liberated nitrous acid. 2 cc. of 20 % sodium bisulphite are added and the solution boiled for about 10 minutes to expel the excess of sulphur dioxide, fragments of broken triangles being added to prevent bumping.

Oxidation to iodic acid. On cooling, from 5–10 drops of bromine are added, and the flask and contents well shaken until the bromine imparts a distinctly brown colour to the solution. Care should be taken not to let the volume get too low during the previous boilings.

The solution is again boiled for about 15 minutes to expel the excess of bromine. Great care must be taken to get rid of the last traces of bromine, and to ensure this a pinch of salicylic acid is added before the solution is cooled down, previous to titration. The unglazed porcelain is removed from the flask and the solution cooled.

Titration. The potassium iodide is added in the manner already described. The liberated iodine is then titrated with a solution of approximately $N/200$ sodium thiosulphate, using a 1 % solution of soluble starch as indicator.

$$\frac{\text{Thiosulphate used} \times \text{iodine equivalent of thiosulphate solution}}{\text{a}}$$

= total iodine in the amount of substance taken for estimation.

The strength of the thiosulphate solution alters from day to day and its iodine equivalent must be determined before each set of titrations. This is conveniently done by titrating it against a solution of potassium bi-iodate (KIO_3HIO_3) which is a stable solution, the iodine equivalent of which has previously been determined [Kendall, 1920].

The accuracy of the Kendall method is illustrated by the following figures. To 5 g. of a dried pasture in which the complete absence of even a trace of iodine had previously been determined, definite amounts of iodine were added.

Estimations were then carried out and the iodine recovered is given in column 2.

g. Iodine added as KI to 5 g. of pasture	Iodine recovered, g.
·00005	·000049
·0001	·000106
·0002	·000200
·0003	·000302

Amounts of iodine as shown were added to 5 g. samples of another pasture taken from land which had been manured with seaweed. More iodine was recovered than was added and it is concluded that this pasture contains about 0·001 % of iodine.

Iodine added, g.	Iodine recovered, g.	Iodine in 5 g. sample of dried grass, g.
·00005	·000098	·000048
·0001	·00015	·00005
·0002	·000248	·000048

We wish to acknowledge our indebtedness to Dr J. B. Orr, who suggested this investigation, and to Mr W. Godden, for helpful criticism.

REFERENCES.

Bohn (1916). *J. Biol. Chem.* **28**, 375.
Cameron (1914). *J. Biol. Chem.* **18**, 335.
Fellenberg (1923). *Biochem. Z.* **139**, 444.
Forbes (1916). *Ohio Agr. Exp Stat. Bull.* 299.
Hanzlik (1910). *J. Biol. Chem.* **7**, 459.
Hunter (1910). *J. Biol. Chem.* **7**, 321.
Kendall (1912). *J. Amer. Chem. Soc.* **34**, 894.
—— (1914). *J. Biol. Chem.* **19**, 251.
—— (1920). *J. Biol. Chem.* **43**, 149.
Krauss (1915). *J. Biol. Chem.* **22**, 151.

CXXVIII. THE YELLOW PIGMENTS OF AUSTRALIAN ACACIAS.

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The golden bloom of Australian Wattles, so favoured as the national flowers by Australian artists and poets, has afforded rich material for the study of the yellow pigments.

The Acacias constitute, with the Eucalypts, a large part of the unique endemic flora of Australia; more than 300 species are distributed throughout the continent, and in all seasons of the year one or more is to be seen in flower. The beautiful florescent masses consist largely of dense crowds of stamens, and their colour ranges from palest cream to deepest golden yellow.

Hitherto nothing was known of the chemical nature of these golden wattle-blossoms, or of the substances to which they owed their beauty. In this investigation the yellow pigments have been extracted, and their properties studied.

Plant pigments are considered biochemically in two distinct groups—the plastid pigments and the water-soluble pigments. In the former the molecule of the colour substance is combined in some obscure chemical way with the other components of the complex colloidal molecule of protoplasm; in this group chlorophyll, carotene, and xanthophyll are well known. Of the water-soluble pigments found in solution in the cell-sap, the yellow coloured substances are mostly flavone and xanthone derivatives, while the blue, red, and purple colours of flowers belong to the group of anthocyanins.

EXPERIMENTAL.

The Material. Four typical examples of Acacias were chosen for this investigation:

I. *Acacia discolor.* II. *Acacia linifolia.* III. *Acacia decurrens*, var. *mollis.* IV. *Acacia longifolia.*

Two of these possess the characteristic pinnate mimosa leaves, and two have their leaves replaced by phyllodes in response to their xerophytic environment. The flowers were collected in quantity at the period of maximum inflorescence, in the neighbourhood of Sydney and the Blue Mountains.

General Method of Extraction. The fresh flowers were immersed in small amounts at a time in boiling water, in order to destroy the enzymes, and the boiling continued till most of the soluble colouring matter was removed. The

large volume of fluid was concentrated and filtered from mucilage and coagulated proteins. The solution was then precipitated by lead acetate; the precipitate containing the colouring matter and tannin. The lead was removed, and the pigments were hydrolysed by boiling with sulphuric acid, during which operation the glucosides were decomposed into yellow pigment and sugars. The colour was extracted with ether, and the remaining aqueous fluid was reserved for the sugar. On distilling the ethereal extract the colouring matter was obtained in the crude state, and was then purified by a series of fractional crystallisations.

I. *Acacia discolor.*

The flowers of *A. discolor*, the sunshine wattle, collected in April, were extracted and treated with ether. The absence of yellow pigment from this ethereal extract showed that no free flavone existed in the plant, and that the pigment was present wholly in the form of a glucoside. The latter was precipitated by lead acetate, and hydrolysed. This yielded (1) a black resinous substance insoluble in the hot fluid; (2) a deep brick-red deposit which formed on cooling; (3) a bright red solution.

The second and third of these gave with ether a yellow pigment. The lead filtrate was further treated with basic lead acetate, hydrolysed, and extracted with ether. The ethereal solutions were then agitated with alkali carbonates from which the yellow colouring matter was precipitated by acid; the alkaline solution was first green in colour, then became red, and changed to purple and violet.

Purification of the crude yellow pigment. The yellow crystalline deposits contained a red and an amorphous brown substance intermixed. By dissolving in hot alcohol a deep red solution resulted, and on carefully diluting this with hot water a point was reached when the yellow pigment could be crystallised out alone. When recrystallised in this way a number of times, the filtrate at last ceased to be red, and a mass of lemon-yellow, needle-shaped crystals was obtained. These possessed an almost constant melting point, and were submitted to a rigid investigation to determine their properties and constitution.

Products obtained from the above operations. 1200 g. of fresh flowers gave:

24 g. black resinous substance.

32 g. of brick-red deposit after hydrolysis, consisting of phlobaphenes.

2·4 g. of crude yellow pigment.

A solution containing the glucosidal sugars.

A brownish-yellow amorphous substance from the mother-liquors after crystallisation of the yellow pigment.

Deep crimson-coloured aqueous solutions exhibiting the properties of anthocyanins and tannins.

A viscid oil of strong acrid odour, obtained from the ether extract after the yellow pigment had been removed by alkali.

(*a*) *The soluble Yellow Pigment.*

In order to complete the purification of the yellow substance the whole was converted into its acetyl derivative. The compound separated from water as a brown crystalline mass, and after purifying with charcoal and recrystallising from hot alcohol, a pure white product was obtained. The acetyl derivative possessed a constant melting point, and was afterwards quantitatively hydrolysed. The yellow pigment obtained in this way was now assumed to be pure.

(1) *Properties.* The substance appeared under the microscope as beautiful masses of crystals, fine hair-like needles of a pale yellow colour, possessing a melting point of 274°. By adding a drop of ammonia to 10 cc. of a solution containing the smallest speck of the substance a deep yellow colour was produced; this is a characteristic reaction of flavones, which have little colour of their own in the glucosidal condition.

Lead acetate produced a bright orange-red precipitate.

Ferric alum in alcoholic solutions gave a purple colour.

Sulphuric acid dissolved the pigment to a bright yellow solution which, on standing some hours, exhibited a bluish-green fluorescence.

These indications that the substance belonged to the flavone group were confirmed by the following quantitative results.

(2) *Sulphuric acid derivative.* The substance, in acetic acid solution and under anhydrous conditions, united with sulphuric acid and yielded a mass of bright orange-red crystals in the form of minute thin needles. These when purified were found to decompose quantitatively, yielding the original substance.

0·1695 g. compound gave 0·1262 g. pigment = 74·45 %
0·0704 g. „ „ 0·0524 g. „ = 74·43 %
74·45 % of pigment represents a Mol. Wt. of 285·8
Calc. for $C_{15}H_{10}O_6$ 286

Five different flavones are known possessing this formula.

The property of combining with mineral acids in this way is probably a general reaction for the yellow colouring matters of the pheno-γ-pyrone class, as has been pointed out by A. G. Perkin. This property has been shown to be due to the oxygen atom in the pyrone ring becoming quadrivalent, and is also said to be characteristic of the γ-pyrone ring.

(3) *Disruption of the Pyrone Nucleus.* In order to determine the nature of the benzene rings attached to the pyrone nucleus, the yellow compound was submitted to fusion with potassium hydroxide at 200°. From the product, phenols and acids were separated. In the former, phloroglucinol was identified by its reactions and by the crystals of the tribromo derivative. The solution of organic acids gave with ferric alum a bluish-green colour, which changed to violet with sodium acetate, and to red with sodium carbonate; but no coloration was produced by a ferrous salt. Protocatechuic acid gives these colour reactions; but it also gives a violet colour with ferrous sulphate.

The small amount of substance left, when recrystallised, separated in radiating clusters of white needle-shaped crystals, melting at 192°. The discrepancy could not be removed at this stage, as the weight of material was quite inadequate for the preparation of salts or the molecular weight determination.

Later, a probable explanation was found in a paper by Barth and Hlasiwetz [1865]. Here protocatechuic acid was shown to form with *p*-hydroxybenzoic acid a compound of constant composition and a constant melting point of 192°. Ferric chloride, acting on the latter acid alone, produced a yellow colour, while the mixture gave the green of the former acid.

The results obtained by fusion of the yellow pigment with potash indicate, therefore, the presence of phloroglucinol, *p*-hydroxybenzoic acid, and protocatechuic acid, among the products of decomposition.

The highly purified pigment was not used for this experiment: it was reserved for the quantitative determination of acetyl and sulphuric derivatives. The sample of pigment used for the alkali fusion, from which it is not recoverable, consisted of the second and third crops of crystals, and although much time was spent in its purification it was subsequently found still to give faint colour reactions of tannins. In the later examination of the tannins protocatechuic acid was identified; and this is a possible source of the acid in the decomposition of the yellow pigment, since one only of these two acids can belong to the flavone molecule.

(4) *Acetylation.* The acetyl compound was prepared and purified. It consisted of fine, white, needle-shaped crystals of silky lustre, matted together into a network; these were anhydrous after drying at 130°, and melted at 179°.

The number of acetyl groups in this compound was then determined by hydrolysis with sulphuric acid, and the yellow pigment thus set free was deposited in its original form.

0·0327 g. acetyl derivative gave 0·0201 g. pigment = 61·5 %
1·3100 g. „ „ „ 0·8075 g. „ = 61·6 %

In a substance of molecular weight 286, this result represents four acetyl groups. The original substance therefore contains four hydroxyl groups in its molecule.

Kaempferol contains four (OH) groups, and tetra-acetyl kaempferol melts at 181°. (See Fig. 2, p. 964.)

(5) *Methoxy-groups.* The testing for CH_3O-groups was carried out by Perkin's modification of Zeisel's method, but no methyl iodide was evolved, and the pigment was recovered unchanged from the mixture. Methoxy-groups are therefore absent from the molecule of the substance.

(6) *Oxidation.* The substance dissolved in dilute soda to a yellow solution. From this liquid the pigment could at first be reprecipitated by acid, but when a current of air was aspirated through the solution for 24 hours, a profound change was noted: the colour became dark brown, and the pigment, which was no longer precipitated by acid, could not be detected in, or recovered from,

the solution. Evidently it had been decomposed by oxidation and, when agitated with ether, a crystalline substance was obtained, which gave the reactions of phloroglucinol.

The nature of this result was shown by Perkin to be due to a particular hydroxyl group in the α position of the pyrone nucleus, the presence of which in a flavone molecule characterises the subgroup of the flavonols.

(7) *Metallic Derivative.* The attempt to prepare a flavonol potassium salt was unsuccessful. A very dark precipitate formed which settled as an Indian-red coloured granular powder, the superfluid being deep red. This granular deposit showed the same melting point as the yellow pigment. The typical orange prisms said to be obtained from the flavonols could not be prepared in this case, and one may conclude that the acidic properties of the substance in question are so feeble that the compound with a metal is difficult to form.

(8) *Conversion of Flavonol to Anthocyanidin.* When we compare the molecular configuration of yellow flavonols with red anthocyanidin pigments, it is observed at once that the difference, though fundamental, is very slight. Theoretically, in passing from one to the other, the change takes place in the 1:4 positions of the pyrone ring, where the oxygen atom changes in valency from 2 to 4, and the CO group is reduced to CH.

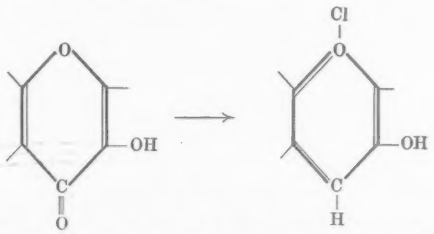

Fig. 1. Pyrone nucleus showing change from flavonol to anthocyanidin.

Such a change was effected practically by treating the alcoholic solution of the yellow pigment with nascent hydrogen. The colour at first became green, then changed suddenly to a brilliant red.

This product may be described as an artificial anthocyanidin, a reduced oxonium salt from flavonol. It exhibited all the general properties of anthocyanidins. When the red alcoholic solution was exposed to air and light it soon became colourless, owing to some allotropic change. When very dilute ammonia was added to the fresh red solution, a blue colour appeared, and with the decolorised solution yellow was formed, the other stages giving mixed green reactions due to both forms. When the red solution was agitated with acid and amyl alcohol the red pigment passed into the latter solvent. The red amyl solution was separated and, on the addition of a drop of sodium carbonate, a change of colour was observed, the solution passing rapidly from blue to green. Finally the alkaline amyl solution was shaken up with water, when the red anthocyanidin suddenly went back into the aqueous solution.

(*b*) *The Sugar of the Glucoside.*

During the process of extraction of the flavonol it was shown that the latter was present as a glucoside. The sugar resulting from the hydrolysis was isolated as a mass of white crystals. In the examination of these galactose was proved absent. The colour tests indicated the presence of pentose and when the crystals were boiled with sulphuric acid, methylfurfural was evolved, which gave the yellow aniline acetate test, changing to red with acid.

The sugar, thus shown to be a methylpentose, was subsequently proved to be rhamnose from the properties of its osazone.

The osazone crystals were purified by six crystallisations from dilute alcohol, and then fractionated. The first and last fractions had melting points of 187° and 188° respectively. There was therefore only one compound present.

The melting point of rhamnose has been recorded by E. Fischer as 185°, and by Votoček as 186°–187°.

(*c*) *The Tannins.*

A large amount of a reddish-brown granular substance was deposited on cooling the hydrolysed aqueous extract of the flowers; gallic acid also crystallised out from this solution. The dry red powder was fused with potash at 200°, and from the acidulated aqueous solution phloroglucinol and protocatechuic and gallic acids were isolated.

The red substance is therefore the phlobaphene or red anhydride of a tannin of the catechol group. Gallic acid is generally regarded as indicating the presence of a gallo-tannin, but A. G. Perkin has recorded a rare exception in the tannin of *Pistacia*, a small European tree (mastic tree), which gave both a red phlobaphene of the catechol series, and also gallic acid and phloroglucinol after fusion. Gallic acid also exists free in certain plants. The results indicate either the presence of two different groups of tannins—a gallo-tannin and a catechol tannin—or one similar to the rare instance mentioned, which appears not to coincide with the present classification of the tannins.

The deep red-coloured fluids obtained all through this work owe their colour to a certain extent to the phloroglucinol in the tannin molecule. During the acid hydrolysis, pentose sugars are formed from various sources such as lignin, pentosans, and the rhamnose glucoside. These give the phloroglucinol red when heated with acid.

(*d*) *The Plastid Pigments.*

Two different tints of yellow may be observed in the inflorescence of *Acacia discolor* when in full bloom: the anthers possess a shining golden yellow colour, while the long filaments are pale cream. If fresh flowers be submitted to the action of ammonia vapour the flavone reaction at once appears, and the previously pale parts of the stamens assume the characteristic deep yellow tint. If some cut flowers be laid aside for a few days and afterwards examined with a lens, the withering is seen in the pale filaments which have become brown,

while the bright yellow of the anthers persists. The brown portions now respond much less to the action of ammonia, for probably the flavonol pigment has been partly oxidised. The unchanged golden yellow parts are most probably those in which the colour is fixed in the chromoplasts as carotene and xanthophyll.

The latter were isolated from the flowers by first removing the water-soluble pigments, and saponifying the chlorophyll in cold alcoholic potash. Carotene and xanthophyll were then obtained by extracting with ether. The weight was equivalent to 0·9 % of the flowers (dried at 100°).

The solution of carotene and xanthophyll showed the complete absorption of the violet and blue rays from λ 495$\mu\mu$.

II. *Acacia linifolia.*

The pale cream-coloured flowers of this species were collected in June, and treated as in the previous case.

From 2 kilos. of flowers about half a kilo. of phlobaphenes was obtained. The yellow pigment was similar to that described above, and the yield was 0·17 % of the air-dried material. The plastid pigments formed 1 %, and an approximate separation gave 0·75 % of carotene, and 0·25 % of xanthophyll.

III. *Acacia decurrens.*

The golden yellow flowers were collected in December, and yielded the same pigment in small amount.

Gallic acid was obtained here, as in *A. discolor*, in considerable quantity, along with the phlobaphene of the catechol tannin.

IV. *Acacia longifolia.*

The deep yellow flowers of the "Golden Wattle," collected in January, yielded the same pigments. The ethereal extracts of this species presented some peculiarities not observed in the previous extracts: for instance, in place of the bright yellow coloured flavone solutions of the three species, this one gave a solution which was quite colourless by transmitted light, while by reflected light it appeared brilliant purple, and on evaporating the solvent the yellow pigment separated.

Conclusions.

The yellow pigments isolated from the flowers of the Acacias gave the following data:

	Acacia discolor	*A. linifolia*	*A. decurrens*	*A. longifolia*	Kaempferol
Melting point of flavonol	274°	275°	274°	276°	274°
" " acetyl-flavonol	179°	180°	181°	181°	181°
Amount of flavonol	·08	·07	·006	·06 % of fresh flowers	
Carotene, xanthophyll	·27	·33	—	·14 " "	

The water-soluble pigment is proved to be a flavonol glucoside of kaempferol and rhamnose.

It is noteworthy that *A. linifolia*, with the palest yellow flowers, contains the largest amount of carotene and xanthophyll, while less than half the amount was obtained from the deep golden yellow flowers of *A. longifolia*.

In conclusion, the author desires to express his indebtedness to Professor H. G. Chapman for affording laboratory accommodation and facilities for carrying out this research.

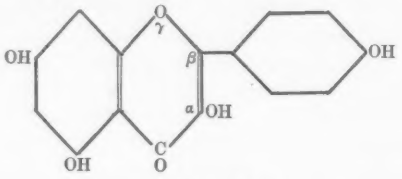

Fig 2. Kaempferol.

Summary.

Four different species of Australian acacias have been examined to ascertain the nature of the colouring matter of their yellow inflorescence.

The water-soluble yellow pigments isolated from each species were identical, and were proved to be a glucoside of kaempferol. The kaempferol consisted of fine hair-like needles of a lemon-yellow colour. In exceedingly dilute solutions alkali produced a very deep yellow colour, and in sulphuric acid the solution exhibited a bluish-green fluorescence.

An oxonium salt was formed with sulphuric acid, of bright orange crystals, from which by quantitative decomposition the molecular weight of the pigment was obtained as 286.

By fusion of the pigment with potash, phloroglucinol, and *p*-hydroxybenzoic and protocatechuic acids were identified, the two acids being combined as a compound of constant composition.

The kaempferol exists in the flowers as a rhamnose glucoside: no free flavonol was found.

The yellow flavonol could be transformed by nascent hydrogen to a red solution, resembling the anthocyanins.

The acacia tannins were composed of phloroglucinol and protocatechuic and gallic acids, and deposited on hydrolysis large amounts of the red phlobaphene anhydrides.

The carotene and xanthophyll as plastid pigments were present in amounts of from 0·14 to 0·3 %, and the flavonol of about 0·06 % of the fresh flowers.

Reference.

Barth and Hlasiwetz (1865). *Liebig's Annalen*, **134**, 276.

CXXIX. A COLORIMETRIC METHOD FOR THE ESTIMATION OF SUGAR IN BLOOD.

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Introductory.

With the recent introduction of insulin in the treatment of diabetes, the necessity for methods of determining accurately the sugar in small quantities of blood has increased. For clinical purposes, colorimetric methods have found favour, owing to their reasonable accuracy and comparative ease of manipulation. Most of the methods which have been used for the colorimetric estimation of sugar in blood have involved the production of colour by the reduction of some aromatic organic compound.

Wacker [1910] proposed *p*-phenylhydrazinesulphonic acid and caustic soda as a colorimetric reagent, whilst Lewis and Benedict [1915] employed picric acid and sodium carbonate as a reagent, the glucose in the blood, if of sufficient concentration, reducing it to the highly coloured salt of picramic acid at a temperature of 100°. Pearce [1915] showed that the reduction could be carried out in more dilute solution, if the higher temperature of an autoclave were employed.

Although the Lewis-Benedict method found great favour at first, and displaced most of the older methods, it was soon recognised that it gave results higher by about 30–50 %, than those obtained by other methods. Hunter and Campbell [1917], de Wesselow [1919] and others have shown conclusively that the method is unreliable on the following grounds:

1. Picric acid is readily reduced, under the conditions used, by substances in the blood other than sugar. Thus it is reduced by such substances as creatine, creatinine, and uric acid, so that the results are higher than those obtained by a titrimetric method devised by MacLean [1919]. This would not be of so much importance as would at first appear, if the results were consistently high, but the quantity of these other reducing substances in blood varies probably as much as the sugar content.

2. The colorimetric comparison is reliable only when the intensities of the tints employed are approximately the same.

Sumner [1921] introduced 3–5-dinitrosalicylic acid as a new reagent for the colorimetric determination of sugar in urine. He pointed out that, unlike

picric acid, this substance is not reduced by creatine, creatinine, or acetone, but that it is reduced by uric acid and other urinary constituents.

It has been found that the sodium salt of dinitrosalicylic acid is a sensitive reagent for the colorimetric estimation of sugar in blood, and it will be shown that the errors due to other reducing substances present are for practical purposes negligible. Moreover, it has been found possible to work accurately with 0·5 cc. of blood, even when carrying out the colour comparison in 25 cc. Nessler cylinders.

The reducing action of creatinine was tested carefully. In some experiments the colour produced under standard conditions by 1 mg. of glucose, was compared with that obtained with the same weight of glucose and ten times the maximum quantity of creatinine likely to be present in 1 cc. of blood. The increase in the results due to the presence of creatinine was not more than 4 %.

In a similar series of experiments the maximum amount of uric acid to be found in 1 cc. of human blood was used. The colour obtained with 1 mg. of glucose and this quantity of uric acid, was never more than 2 % deeper than that obtained from 1 mg. of glucose alone.

It was found essential to compare colours of approximately the same depth of tint, that is, within 25 % of each other, but it is advisable to have them as nearly equal as possible. When the coloured solution was examined by means of a spectrometer it was found that there were no well-defined absorption bands in the visible spectrum but that the solution cut out the whole of the violet and blue and part of the blue-green. As the solution was diluted it cut out less and less of the blue-green and blue.

Lastly, it may be added that unlike many of the other colorimetric methods, no error due to back oxidation through atmospheric oxygen, is involved.

Experimental.

Preparation of 3–5-dinitrosalicylic acid. In the preparation of dinitrosalicylic acid, the nitration of salicylic acid was carried out as recommended by Sumner, although the purification was effected in a slightly different manner. 75 g. of concentrated sulphuric acid were mixed with 15 g. of concentrated nitric acid and the mixture cooled to 0°. 15 g. of dry powdered salicylic acid were added gradually, the mixture being kept well cooled. The whole was poured into water, and after solidification, filtered and washed once with a little cold water. The purification was not carried out by the method of Sumner involving the preparation of the sodium salt, as it was found possible to free the dinitrosalicylic acid from accompanying mononitro acids by extracting the mass with cold water. On evaporating down the filtered extract, crystals of pure dinitrosalicylic acid separated out. The crystals were then dried at a moderate temperature, and their purity determined by their M.P. (173°).

Preparation of reagents. Three standard solutions are required in addition to "dialysed iron" and saturated sodium sulphate solution. It is doubtful whether dialysed iron can be obtained free from chlorine. Specimens of

dialysed iron (B.D.H., B.P. strength) were found to contain appreciable amounts of chlorine, although they gave no precipitate with silver nitrate. In testing for chlorine the following procedure was adopted. To the dialysed iron a few drops of a solution of silver nitrate in dilute nitric acid were added. The solution was then made alkaline with ammonia, and the precipitated ferric hydroxide filtered off. The filtrate invariably gave a precipitate of silver chloride when acidified with nitric acid. All attempts to obtain dialysed iron free from chlorine led to the precipitation of the colloid, before a negative test for chlorine could be obtained. Since the dinitrosalicylic acid gives a red colour with ferric chloride, this is of importance, as the possibility of this affecting the colorimetric estimation of the sugar in the blood filtrate must be guarded against by the use of an excess of alkali in the colour reaction. The three standard solutions are: (1) 3 % caustic soda solution; (2) glucose solution. It is convenient to make up a solution from pure glucose, containing about 2 mg. per cc., which can be standardised accurately against Fehling's solution. Such a solution keeps unchanged for a long time, in the presence of toluene, and can be diluted 1:2 or 1:4 according to the intensity of colour required in the standard. (3) Dinitrosalicylic acid solution. This is prepared according to the directions of Sumner. 2 g. of dinitrosalicylic acid are dissolved in about 70 cc. of hot water containing 10 cc. of 20 % sodium carbonate solution. The solution is cooled and made up to 100 cc. If the laboratory temperature falls too low, the salt tends to crystallise, but the crystals redissolve on warming.

Procedure. 1 cc. of blood is measured carefully by means of an accurate pipette into a 50 cc. flask. The pipette is rinsed free from blood with distilled water into the flask. 2 cc. of saturated sodium sulphate solution are added, and sufficient water to make up the volume to about 25 cc. The flask is heated for a few minutes in a boiling water-bath, until the protein is coagulated, when 10 cc. of dialysed iron (sp. gr. 1010) are added. The flask is cooled and the volume made up to 50 cc. with the addition of one drop of alcohol to disperse the froth. The liquid is filtered through a Whatman filter paper, and the filtrate used for the estimation.

25 cc. are measured out and run into a 50 cc. beaker, which is suspended by means of a ring, in a boiling water-bath, until all the water is evaporated. The white residue is washed down from the sides of the beaker with 1 cc. of distilled water, and 1 cc. of 3 % caustic soda solution, after which 1 cc. of the dinitrosalicylic acid solution is pipetted into the beaker. The beaker is immersed for exactly five minutes in a boiling water-bath, and the resulting solution quickly diluted with cold water, and filtered through glass wool into a Nessler cylinder. The glass wool is washed free from colour, and the filtrate made up to a suitable volume (50 cc.). Blank experiments showed conclusively that there was no absorption of colour during the filtration through glass wool.

The standard, which may be prepared simultaneously, is obtained in the following way: 1 cc. of glucose solution containing 0·5 mg. of glucose is measured into a similar 50 cc. beaker; 1 cc. of caustic soda solution and 1 cc. of dinitro-

salicylic acid solution are added, and the beaker immersed for five minutes in a similar boiling water-bath. The solution is diluted and made up to 50 cc. in a Nessler cylinder as before, omitting the filtration.

If the sugar is present in the blood to a normal extent, the two solutions are comparable in tint, and the colours can be compared in the usual way, by removing a measured quantity of the solution with the deeper tint, until the tints are matched. When carrying out a large number of estimations, it is convenient to arrange the standard in such a manner that its tint is always slightly lighter than that of the unknown solution; so that the same standard can be used many times. If the percentage of sugar in the blood be higher than normal, it is convenient to prepare the standard from 1 cc. of glucose solution containing 0·1 % of glucose. If the blood contain more than 0·3 % glucose, it is best to dilute to 100 cc. as the tint is then too deep at a dilution of 50 cc. for accurate comparison. The standard is then prepared from 2 mg. of glucose. A series of standards may be prepared, and can be kept for at least two days without any visible change of colour. When an estimation is carried out with 0·5 cc. of blood, the volume should be made up to 25 cc., and 12·5 cc. evaporated, after which the procedure is the same. The colours can be compared accurately in suitable 25 cc. Nessler cylinders.

Effect of reducing substances other than sugar present in blood.

1. *Creatinine.* 1 cc. of glucose, containing 1 mg. of the sugar, was heated for five minutes with 1 cc. of caustic soda and 1 cc. of dinitrosalicylic acid solution, and the colour compared with that obtained in a similar experiment wherein 2 mg. of creatinine were added. When the tints of the two solutions were compared in Nessler cylinders, it was found that that of the solution containing the creatinine was deeper only to the extent of 4–5 %. This value was never exceeded in a number of experiments, and therefore the error due to creatinine is negligible.

2. *Uric acid.* A solution containing 0·35 g. of uric acid per litre was made with the addition of a little caustic soda. 1 cc. was measured out into a beaker and evaporated to dryness. 1 mg. of glucose was then added, and the colour obtained by the ordinary procedure compared with that obtained by the use of 1 mg. of glucose alone. The following figures represent the number of cc. of the two solutions when matched after making up to 50 cc.

0·35 mg. uric acid + 1 mg. glucose	1 mg. glucose
cc.	cc.
48	50
49	50
49	50
49·5	50

Average difference, 2 %.

Examples of Sugar Estimations in Blood.

The following are some of the results obtained by the above method with different specimens of blood, all of which were laked with sodium fluoride.

Source of blood		Result with 2 cc.	1 cc.	0·5 cc.
Ox	1	0·070 %	0·076 %	—
„	2	0·069	0·073	—
Normal human	3	—	0·10	—
		—	0·094	—
„ „	4	—	0·088	—
		—	0·090	—
„ „	5	—	0·078	—
		—	0·082	—
Diabetic human	6	—	0·138	0·142 %
		—	0·134	0·138
„ „	7	—	0·121	—
		—	0·119	—
„ „	8	—	0·132	—
		—	0·138	—
„ „	9*	—	0·571	0·570
		—	0·565	0·580

* This specimen was obtained from a patient suffering from acute glycosuria.

Distribution of glucose between serum and corpuscles in ox blood.

A series of estimations of reducing sugar in ox blood, and in the serum derived from the blood, were carried out in order to determine the distribution of the sugar between the serum and the corpuscles. With fresh ox blood and the serum derived from it, the following results were obtained:

	Blood	Serum from blood
1	0·071 %	0·062 %
2	0·067	0·060

After keeping the blood and the serum under aseptic conditions for a fortnight, it was found that the sugar content of the serum had diminished to a very low figure, whereas there had been little change in the sugar content of the blood.

Percentage of glucose in the two specimens of ox blood after two weeks.

	Blood	Serum
1	0·065	0·01*
2	0·063	0·02*

* Too small for accurate estimation.

This result was confirmed by a further experiment. It would seem then, that the conversion of reducing sugar to non-reducing polysaccharide proceeds rapidly in the absence of the corpuscles, which probably retard this change.

With the above method something like half a dozen determinations can be easily carried out in two hours, provided that there is accommodation for evaporating the filtrates simultaneously in separate beakers. It may be added that the evaporation process, which may be regarded by some as an objectionable feature of the method, takes less than three-quarters of an hour, and, given

a suitable water-bath, does not require attention. If desired, the time may be further curtailed by making up in the first place 1 cc. of blood to 25 cc. instead of 50 cc.

It is doubtful whether the measurement of small quantities of blood in a pipette is desirable on the score of accuracy, as the blood adheres slightly to the glass surface and cannot be removed readily by washing with cold water. It is probable that the accuracy of blood sugar estimations would be materially increased if the sugar concentration were expressed in grams per cent. instead of per 100 cc.

Finally I must associate myself with many others in the opinion that the determination of the concentration of reducing sugar may not be an absolute guide in the treatment of diabetes, as it leaves out of account the significance of the non-reducing carbohydrate known to be present in the blood. There is no doubt that an intimate relation exists between the reducing and the non-reducing carbohydrates. The latter have so far received little attention, and it is proposed to study the problem in the near future.

I wish to thank Prof. A. R. Ling and Mr D. R. Nanji for their advice and encouragement in the course of this work.

REFERENCES.

Hunter and Campbell (1917). *J. Biol. Chem.* **32**, 195.
Lewis and Benedict (1915). *J. Biol. Chem.* **20**, 61.
MacLean (1919). *Biochem. J.* **13**, 135.
Pearce (1915). *J. Biol. Chem.* **22**, 525.
Sumner (1921). *J. Biol. Chem.* 47–48, 5.
Wacker (1910). *Z. physiol. Chem.* **67**, 197.
De Wesselow (1919). *Biochem. J.* **13**, 148

CXXX. STUDIES ON XANTHINE OXIDASE.

I. PREPARATION AND PROPERTIES OF THE ACTIVE MATERIAL.

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Morgan, Stewart and Hopkins [1922] have recently demonstrated the existence in milk of an enzyme capable of oxidising the purine bases hypoxanthine and xanthine to uric acid. It has long been known that an enzyme with this property exists in most living tissues, and the work of these authors on several points in connection with the mode of action of the enzyme in milk left little doubt that it is identical with that in the tissues. As is the case with most other oxidising enzymes, the xanthine oxidase[1] is capable of using various oxidising agents to bring about the reaction. The technique mainly used hitherto for the study of oxidising enzymes consists in the measurement of the rate of disappearance of the oxidising agent used, for example, the rate of absorption of oxygen or of decolorisation of added methylene blue.

The investigation of the properties of any individual oxidising enzyme in preparations obtained from tissues is usually complicated by the presence of other enzymes. Many of these are able, with the aid of the above-mentioned oxidising agents, to oxidise various substances present and they thus tend to mask the action of the enzyme under investigation. This effect renders it necessary to control every experiment by appropriate "blank" tests, and makes it a matter of some difficulty to determine the velocity of the single reaction brought about by the enzyme in question.

For the study of the xanthine oxidase milk offers special facilities as a source of the enzyme, as not only are most of the usual interfering enzymes absent, but there is also normally a complete absence of any substances which can be oxidised by those enzymes present in it. A systematic study of an individual oxidising enzyme appears only to have been made in the case of the enzyme converting succinic acid to fumaric acid—the succinoxydone of Batelli and Stern, studied by Ohlsson [1921] and Widmark [1921].

In this class of work one has a choice of two main experimental techniques, both of which were employed by Morgan, Stewart and Hopkins, namely the

[1] Although the enzyme is generally spoken of as the xanthine oxidase, it is not strictly an oxidase, but rather an oxydone in the sense of Batelli and Stern. The usual name has however been retained here.

measurement of the absorption of oxygen by means of the Barcroft apparatus, and the reduction *in vacuo* of methylene blue to its leuco form. The latter method, first used for the study of oxidising enzymes by Thunberg [1920], is much the easier, and it was the only method used by Ohlsson and Widmark. It has been largely used in the present work.

The procedure of Morgan, Stewart and Hopkins was adopted with little modification. The experiments were all carried out in Thunberg vacuum tubes which were exhausted by a water-pump to a pressure of a few mm. of mercury, refilled with nitrogen and again exhausted, the procedure being repeated two or three times. The methylene blue was made up to a strength of 1/5000 in distilled water, and the solutions of the purine bases were made up to a strength of 2 mg. per cc. in very dilute NaOH, as it was found that they could not easily be kept for any length of time in neutral solution. If neutralised and allowed to stand several days a change is found to take place, especially if phosphate is present. They become altogether incapable of oxidation by the enzyme, and at the same time are no longer precipitable by ammoniacal silver nitrate. Although no definite growth of bacteria could be observed, the change appears to be due to bacterial action, since it did not take place if special precautions were taken to keep the solutions absolutely sterile. This observation is obviously of considerable practical importance in carrying out investigations on this enzyme. The addition of the slightly alkaline purine solutions in the small quantities used in most experiments did not appreciably change the p_H of the enzyme solution. When it was necessary to add larger quantities, the purine solutions were so far neutralised that, although there was no precipitation, no appreciable change in the p_H of the enzyme solution occurred when they were added.

The reductions took place in a thermostat kept at 37°.

There are several disadvantages in the employment of milk for the investigation of the properties of the xanthine oxidase. Firstly, different milks vary enormously in their oxidase activities, so that it is difficult from day to day to obtain comparable experiments. Secondly, the activity of any one milk is not usually constant for more than a few hours at a time; the activity falls off fairly quickly, and the milk if sterile usually becomes inactive in the course of one or two days. Thirdly, the milk, if not perfectly sterile, invariably develops a marked power to reduce methylene blue by itself in the absence of purine substances, owing to the growth of bacteria.

Attempts were therefore made to obtain a preparation from which the various complex constituents of milk had as far as possible been removed, and which would contain the oxidase in a stable form.

Upon removal of the fat by centrifuging there was a marked fall in activity of the xanthine oxidase, and this led to the belief that the enzyme was adsorbed on the fat surface. Further investigation, however, showed that the effect was probably due to the catalytic action of the large surface presented by the fat. On adding boiled cream, which of course is itself inactive, to a solution

containing the enzyme, a large increase of oxidase activity was produced. Fat, however, had no effect upon the Schardinger enzyme.

On further investigation it was found that the oxidase was precipitated completely, with the caseinogen, on half saturation with ammonium sulphate. This naturally led to the following routine method of preparation of the active material.

The activity of the milk is first tested in the usual way with a small trace of hypoxanthine and methylene blue. It is important to use as active a milk as possible, as the activity of the final preparation depends entirely upon that of the milk employed. The milk is then mixed with an equal volume of saturated ammonium sulphate solution, and allowed to stand for a few minutes. The precipitated caseinogen and fat are then filtered off and dried as far as possible by pressing between filter-papers. The ease of subsequent manipulation depends to a great extent upon the thoroughness with which this is carried out. The mass is now broken up and extracted thoroughly with cold ether, the process being hastened by frequent shaking. The ether is drained off and the material again pressed between filter-papers so as to remove as much ether and water as possible, after which it is spread out and dried in a vacuum desiccator for about 12 hours. After this it is again extracted with ether, dried thoroughly *in vacuo*, and powdered in a mortar.

The resulting preparation is very convenient to use, consisting of a fine white powder readily soluble in water forming a slightly opalescent solution. It was the only preparation used in this work. The powder is extremely active, and if kept in a well stoppered bottle retains for long periods its activity unimpaired. Preparations usually exhibit a good activity after being kept six months or more, and in all cases seem to remain quite free from any trace of bacterial contamination. The active material consists of caseinogen together with small amounts of ammonium sulphate.

It was found that the preparation contained not only the xanthine oxidase but also the Schardinger aldehydeoxidase. The small amount of adenase found in milk by Morgan, Stewart and Hopkins is also present in the final preparation. Reduction was not obtained in presence of any substance other than hypoxanthine, xanthine, adenine and aldehydes.

Many methods were tried in an endeavour to separate the enzyme from the caseinogen[1]. The concentration of ammonium sulphate used for precipitation was varied, in the hope that the caseinogen might be precipitated first; but as soon as the caseinogen is completely precipitated the remaining solution from which the precipitate has been centrifuged off is quite inactive. (In preparing the active material the addition of at least an equal volume of saturated ammonium sulphate is advisable in order that the final product may be easily soluble in water.) It was then thought that the enzyme might be separated from the caseinogen by adding to the solution a substance more

[1] We have very recently found that when milk is clotted by rennin a considerable part of the enzyme remains in the whey.

strongly adsorbing than caseinogen. Little of the enzyme is removed from neutral solution by strongly adsorbing substances, such as animal charcoal and kaolin. Willstätter [1923] found alumina very useful in the purification of certain enzymes, which were adsorbed by the alumina and could then be washed off. Preliminary experiments of this nature have been carried out with the xanthine oxidase. A suspension of freshly prepared alumina was thoroughly mixed with the enzyme solution and then removed by centrifuging. The resulting solution was practically inactive. On testing the alumina with hypoxanthine in the Barcroft apparatus there was found to be a steady but rather slow uptake of oxygen. This shows that the enzyme is still active on the alumina, but as yet we have not been successful in removing it from the adsorbent. Further experiments with this object in view are in progress.

It was observed that by filtering the enzyme solution through filter-paper the activity was greatly reduced. A moderately dilute solution was rendered completely inactive, while a concentrated solution lost about 50 % of its activity. There was, however, no visible residue on the filter-paper. In order to determine the weight of the material removed from solution, a filter-paper was dried in the vacuum desiccator and weighed. Through it was filtered twice a solution of a very active caseinogen preparation containing 1·5 g. in 30 cc. of p_H 7·6 phosphate buffer. The filter-paper was then thoroughly washed with distilled water, dried in the vacuum desiccator and weighed. The increase of weight was 0·0012 g. The filtrate and washings were found to be inactive, while a filter-paper treated in a similar manner was found in the Barcroft apparatus to take up oxygen in presence of hypoxanthine as rapidly as an unfiltered solution. The presence of filter-paper renders the methylene blue technique inapplicable, as the methylene blue becomes adsorbed on the filter-paper and is then reduced only with extreme slowness.

The adsorption of the enzyme by filter-paper affords an explanation of the discrepancy between the results of Rohde and Jones [1909] and those of Morgan, Stewart and Hopkins on tissues of the rat. Rohde and Jones, having filtered their tissue extracts, stated that xanthine oxidase was not present in the tissues of the rat, while Morgan, Stewart and Hopkins, working with unfiltered extracts, found that it was present.

The effect of various organic solvents was then tried. Exposure for an hour of the solid preparation to both alcohol and acetone caused a marked decrease in activity, while ether, chloroform, toluene and glycerol had no effect.

It was noticed that dry preparations kept tightly corked up and undisturbed retained their activity better than those which were frequently opened and shaken up. This led to the belief that the enzyme might be undergoing a slow oxidation process brought about by atmospheric oxygen. It has already been observed that milk upon standing loses its activity. This is also true of a solution of the preparation kept in air, whereas a solution kept in an evacuated tube retains its activity unimpaired as long as it can be kept free from bacteria.

SUMMARY.

1. A method for obtaining from milk a stable, water-soluble caseinogen preparation containing the xanthine oxidase in a form suitable for systematic investigation has been described.

2. The enzyme is adsorbed from a neutral solution of the preparation only slightly by animal charcoal, but almost completely by alumina and filter-paper.

3. The enzyme when adsorbed on alumina or filter-paper still retains its activity.

4. The presence of fat in a finely divided state, as in milk, greatly increases the rate of oxidation of purine bases by the enzyme.

5. The enzyme is slowly destroyed by atmospheric oxygen.

6. The enzyme is destroyed by alcohol and acetone but not by ether, chloroform, toluene or glycerol.

7. Hypoxanthine and xanthine on standing in neutral solution become incapable of oxidation by the enzyme.

REFERENCES.

Morgan, Stewart and Hopkins (1922). *Proc. Roy. Soc. London*, B, **94**, 109.
Ohlsson (1921). *Skand. Arch. Physiol.* **41**, 77.
Rohde and Jones (1909). *J. Biol. Chem.* **7**, 237.
Thunberg (1920). *Skand. Arch. Physiol.* **40**, 1.
Widmark (1921). *Skand. Arch. Physiol.* **41**, 200.
Willstätter (1923). *Z. physiol. Chem.* **125**.

CXXXI. STUDIES ON XANTHINE OXIDASE.

II. THE DYNAMICS OF THE OXIDASE SYSTEM.

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(*Received July 31st, 1924.*)

In the previous paper we have described a method for obtaining a preparation of the xanthine oxidase in a form suitable for investigation. While a large amount of work on various enzymes has been done, it appears that the thorough systematic investigation of the dynamics of any one oxidase has not been attempted. The chief difficulty in such work is that of obtaining the enzyme in question free from other enzymes and from oxidisable substances. The above-mentioned preparation is satisfactory in this respect and we therefore have employed it in investigating the xanthine oxidase. The methylene blue technique was used throughout.

The reduction velocity-time curve of the reaction with methylene blue was not investigated by Morgan, Stewart and Hopkins [1922], but they believed that the velocity was constant during the course of the reduction. This has been verified by the use of a modification of the colorimetric method used by Dixon and Tunnicliffe [1923]. The amount of methylene blue remaining unreduced at any instant was determined by matching the tint of the reacting mixture against colour standards prepared by adding known amounts of methylene blue to enzyme solution. It was found that, provided traces of oxygen were carefully excluded, the methylene blue disappeared at a constant rate except when reduction was practically complete, when the reduction velocity fell off slightly. If then the "reduction time" is measured from the commencement of the reaction to the time when the colour has reached a very faint standard "end-point," the reciprocal of the reduction time gives a measure of the reduction velocity. In plotting the curves, the reciprocal of the reduction time in minutes has been multiplied, for convenience, by 10,000.

All the curves given in this paper, since they are typical examples of a large number of experiments, can be relied upon. It is not advisable to place too much reliance on the methylene blue technique until the results have been confirmed by repetition.

p_H *of Solution.*

The reaction velocity was found to be entirely independent of the p_H of the solution within wide limits. Phthalate and acetate buffers markedly inhibit the reaction and therefore could not be used in obtaining this curve,

whereas a solution of the enzyme in either borate or phosphate has a reaction velocity equal to that of an aqueous solution of the same p_H. Borate buffers were therefore used from p_H 8·0 upwards, while phosphate buffers were used between 5·8 and 8·0. On the acid side of p_H 5·8 the p_H of the solutions was adjusted to the required value by the addition of dilute acid to a solution of the enzyme in p_H 7·6 phosphate buffer, the amount of acid required being determined by titration of a sample of the solution. During the addition of

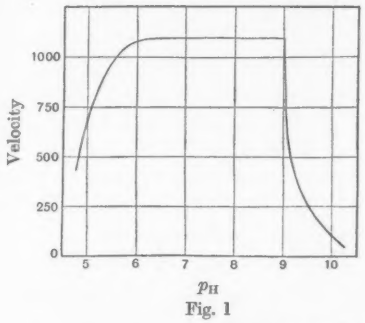

Fig. 1

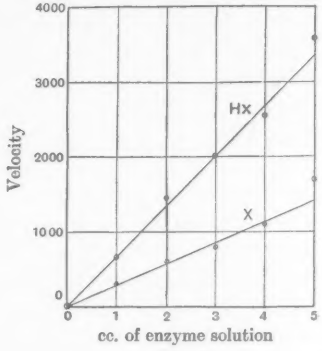

Fig. 2

Fig. 1. *p_H curve with hypoxanthine.*

Each tube contained: 2·5 cc. enzyme solution in water; 2·5 cc. buffer solution; 0·5 cc. methylene blue; 0·2 mg. hypoxanthine.

Fig. 2. *Enzyme concentration curve with hypoxanthine and xanthine.*

Each tube contained: x cc. enzyme solution, $(5-x)$ cc. inactive caseinogen solution (both in p_H 7·6 phosphate buffer); 0·5 cc. methylene blue; 0·2 mg. hypoxanthine or xanthine.

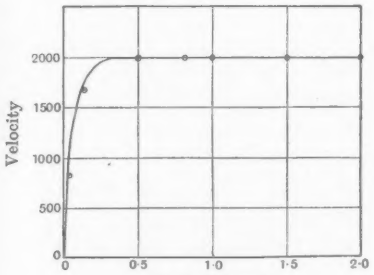

Fig 3

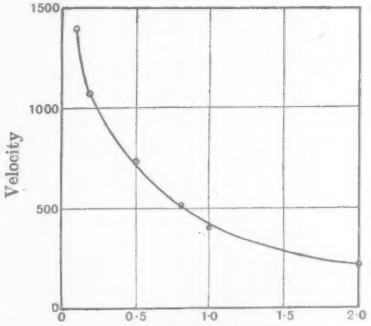

Fig. 4

Fig. 3. *Methylene blue concentration curve.*

Each tube contained: 5·0 cc. enzyme solution (in 7·6 phosphate buffer); x cc. methylene blue; $(2-x)$ cc. water; 0·2 mg. hypoxanthine.

Fig. 4. *Hypoxanthine concentration curve.*

Each tube contained: 5·0 cc. enzyme solution (in 7·6 buffer); 0·5 cc. methylene blue; x cc. hypoxanthine (1 mg. per cc.); $(2-x)$ cc. water.

the acid the solution was thoroughly shaken to maintain the same H ion concentration throughout. Otherwise a partial destruction of the enzyme by the acid might occur. The curve obtained for hypoxanthine is given in Fig. 1.

Those obtained for aldehyde and adenine are similar. The curve shows a constant reaction velocity from p_H 5·8 to 9·0. At 9·0 there is a sudden drop in the reaction velocity, the velocity at p_H 10·0 being only about one-tenth of that at p_H 9·0. The sudden break at 9·0 cannot be due to change of buffer as it comes in the middle of the borate range.

Ohlsson [1921] investigated the p_H curve for the succinoxidase and found a similar break at p_H 9·0. In fact his curve is very similar to that given above for hypoxanthine. The break may possibly be characteristic of this type of oxidising enzyme. Ohlsson found that the position of the break depended on the temperature, the higher the temperature the sooner does the break occur. We have investigated the curve at 15° and at 38°, but at both temperatures the break occurred at p_H 9·0. This break has been found to be due to destruction of the enzyme at reactions more alkaline than p_H 9, since solutions above this p_H do not regain their original activity on readjustment to neutrality. Similar experiments were made to discover the cause of the drop on the acid side of the curve. At p_H 4·2 the activity for hypoxanthine has completely disappeared, but if a solution which has been allowed to stand for 24 hours at p_H 4·2 is then brought back to p_H 7·6 not the slightest loss of activity has occurred. The loss of activity at p_H 4·2 is therefore not due to destruction of the enzyme but to the effect of p_H upon the reaction velocity. The p_H of the solution must be more acid than that at which the reaction can proceed at all before destruction of the enzyme actually occurs. If the solution is brought to p_H 3·5 a complete and irreversible destruction occurs.

Concentration of Enzyme.

With the substrate, methylene blue and caseinogen constant, the concentration of the enzyme was varied. Solutions of equal strength of active and inactive caseinogen were made, and the relative amounts of the two solutions were varied, the total volume being kept constant. The curves obtained with hypoxanthine and xanthine are shown in Fig 2. Exactly similar curves were obtained with the Schardinger enzyme and the adenase.

It will be seen that the reaction velocity is accurately proportional to the concentration of the catalyst. This shows that, throughout the range of concentrations used in any of our experiments, the catalyst is not present in excess, and its concentration is therefore a limiting factor in the reaction. It was subsequently found that the addition of inactive caseinogen had no effect on the velocity of the reaction, so that the relative amounts of enzyme in various solutions can be estimated by determining the reaction velocity under comparable conditions.

Concentration of Methylene Blue

With the substrate and enzyme concentrations and the total volume kept constant, the amount of methylene blue was varied. The reaction velocity, that is, the amount of methylene blue reduced in a given time, is obtained by multiplying the reciprocal of the reduction time by the amount of methylene blue. It will be seen in Fig. 3 that the reaction velocity is quite independent of the methylene blue concentration except when this is very low.

Concentration of Substrate.

With methylene blue and enzyme kept constant, the concentration of hypoxanthine was varied. The curve obtained, which was of an unexpected form, is shown in Fig. 4. The xanthine curve is similar. It will be observed that as the concentration of hypoxanthine is increased the reaction velocity is decreased, the velocity at high substrate concentrations being extremely small.

Morgan, Stewart and Hopkins (unpublished experiments) found that uric acid inhibited the reaction in the case of both hypoxanthine and xanthine. The obvious explanation of this inhibition would be the application of mass action to a reversible reaction. Since, however, a similar inhibitory effect was obtained with hypoxanthine itself, it would seem that the two inhibitions are due to the same cause, and this cannot be mass action in the case of hypoxanthine. It appeared possible that the effect might be due to adsorption by the enzyme of the inhibitory substance, thus preventing the adsorption of one or both of the reactants. The effect might therefore be expected to be given by other purine substances, and in fact guanine, adenine and xanthine itself were found to produce this inhibitory effect upon hypoxanthine. Some of the curves obtained are shown in Fig. 5. The effect, however, seems to be remarkably specific. Caffeine showed no trace of inhibitory effect. The pyrimidine substances uracil, cytosine and thymine were also tried but showed no inhibition. Histidine, which contains the iminazole ring, was also tried, but had no effect.

If hypoxanthine and uric acid inhibit by the same mechanism, hypoxanthine would be expected to produce no further inhibition if added to a solution which already contained enough uric acid to produce the maximum inhibition, that is, a concentration corresponding to a point on the horizontal part of the inhibition curve. The resulting hypoxanthine concentration curve in presence of uric acid is shown in Fig. 6.

It will be seen that the hypoxanthine concentration curve has entirely changed its shape, and now assumes the form of a typical substrate curve, such, for instance, as that found in the case of succinoxidase by Widmark [1921]. This furnishes a proof that the uric acid and xanthine inhibitions are essentially the same in nature.

Critical Concentration.

The initial parts of the hypoxanthine and xanthine concentration curves were then investigated more thoroughly, and the results are plotted on an extended scale in Fig. 7.

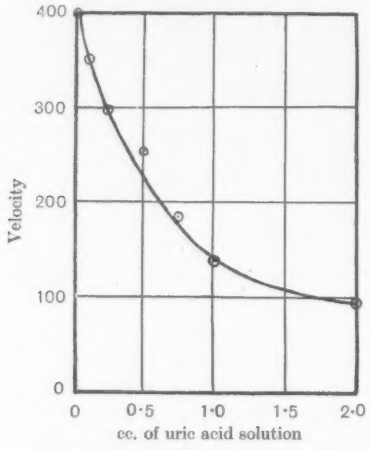

Fig. 5*a*

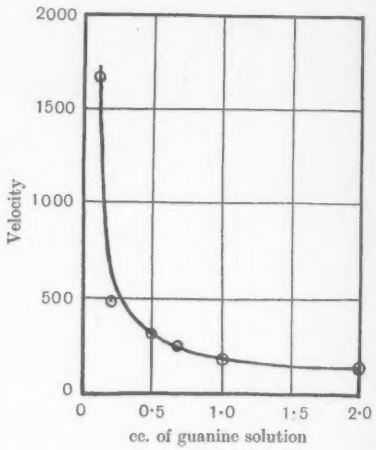

Fig. 5*b*

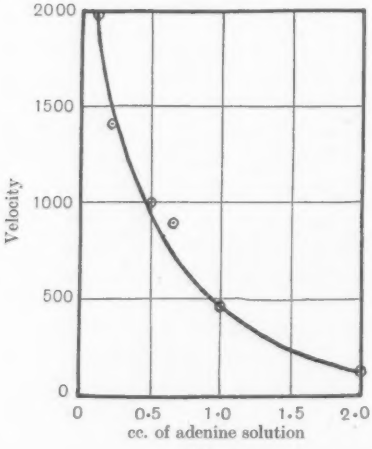

Fig. 5*c*

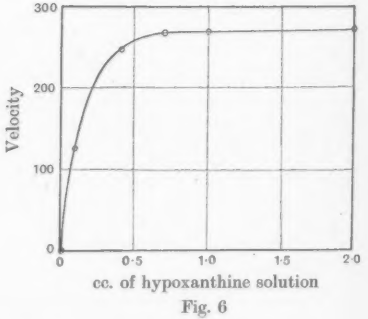

Fig. 6

Fig. 5. *Inhibition of hypoxanthine reaction by (a) uric acid, (b) guanine, (c) adenine.*
Each tube contained: 5·0 cc. enzyme solution (in buffer); 0·5 cc. methylene blue; 0·2 mg. hypoxanthine; x cc. (*a*) uric acid (5 mg. per cc.), (*b*) guanine (1 mg. per cc.), (*c*), adenine (1 mg. per cc.); $(2-x)$ cc. water.

Fig. 6. *Hypoxanthine concentration curve in presence of uric acid.*
Each tube contained: 5·0 cc. enzyme solution (in buffer); 0·5 cc. methylene blue; 2·0 cc. uric acid (5 mg. per cc.); x cc. hypoxanthine (1 mg. per cc.); $(2-x)$ cc. water.

It will be observed that once the substrate concentration is sufficient to reduce the methylene blue completely, the velocity is unaffected by change in concentration until a certain critical concentration is reached, after which inhibition suddenly commences. The critical concentration for xanthine is slightly higher under the same conditions than that for hypoxanthine.

It was found that the critical hypoxanthine concentration, while being independent of the methylene blue concentration, varied with varying enzyme concentration. The more concentrated the enzyme solution, the more hypoxanthine is necessary before the critical concentration is reached. The initial flat part of the curve is thus extended. Morgan, Stewart and Hopkins, using a very active milk, that is, one in which the enzyme is at a high concentration, obtained a constant velocity with varying substrate concentration. Our results show that their range of substrate concentrations lay entirely below the critical point for so active an enzyme solution. It was thought that the effect of changing enzyme concentration might be due to changing caseinogen concentration, but it was found that the addition of an equal amount of inactive caseinogen had no effect on the critical concentration, and the change in the position of the critical point must therefore be due to the change in enzyme concentration. The addition of fat was without effect.

On investigating the curve of uric acid inhibition on hypoxanthine, using a stronger enzyme solution and a smaller quantity of hypoxanthine, it was found that this curve also showed an initial horizontal portion and a critical concentration of uric acid. It should be noted that the curve given in Fig. 5 (*a*) was obtained with a dilute enzyme solution and with a moderate quantity of hypoxanthine already present, and consequently does not exhibit the critical concentration effect. On increasing the enzyme concentration, however, the initial flat part of the curve becomes well marked.

With the hypoxanthine concentration such that inhibition is practically complete, the concentration of methylene blue was varied. The curve is shown in Fig. 8.

Whereas with low hypoxanthine concentration the reaction velocity was found to be independent of methylene blue concentration, it will be seen that with high hypoxanthine concentration the reaction velocity increases with increasing methylene blue. The significance of this will be discussed later.

Morgan, Stewart and Hopkins found that the rate of reduction of methylene blue by hypoxanthine was twice the rate of reduction by xanthine. In certain experiments we were unable to obtain this two to one ratio. Our failure to repeat their results led us to investigate the conditions under which the two to one ratio held. As long as the concentrations of hypoxanthine and xanthine remain below the critical values, the ratio remains accurately two to one, but when the concentration exceeds the critical value the ratio begins to approach, and finally reaches, one to one. In very high concentrations the curve for xanthine crosses that for hypoxanthine, so that xanthine actually reduces methylene blue at a higher rate than does hypoxanthine. Typical curves are shown in Fig. 9.

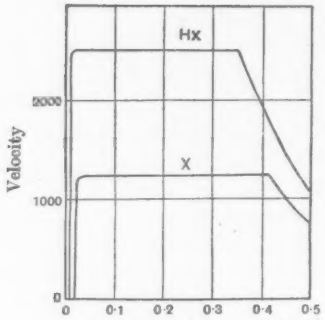

Fig. 7

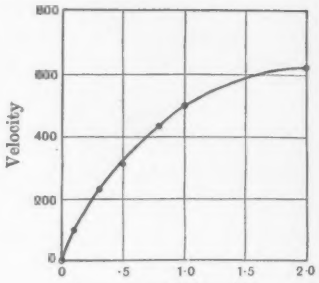

Fig. 8

Fig. 7. *Initial part of hypoxanthine and xanthine concentration curves.*

Each tube contained: 5·0 cc. strong enzyme solution (in buffer); 0·5 cc. methylene blue; x cc. hypoxanthine or xanthine (2 mg. per cc.); $(0{\cdot}5 - x)$ cc. water.

Fig. 8. *Methylene blue concentration curve at high hypoxanthine concentration.*

Each tube contained: 5·0 cc. enzyme solution (in buffer); 1·0 cc. hypoxanthine (2 mg. per cc.); x cc. methylene blue; $(2 - x)$ cc. water.

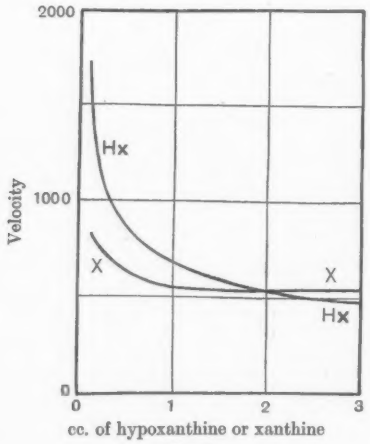

Fig. 9

Fig. 9. *Hypoxanthine and xanthine concentration curves compared.*

Each tube contained: 4·0 cc. enzyme solution (in buffer); 0·5 cc. methylene blue; x cc. hypoxanthine or xanthine (2 mg. per cc.); $(3 - x)$ cc. water.

Discussion.

It will be convenient if the experimental results which must be explained by a complete theory of the mechanism of the enzyme action are summarised here. They are as follows:

1. With high concentration of purines the velocity is diminished.
2. There is, however, an initial range of concentration through which the velocity remains constant.
3. There is a definite concentration of purines at which inhibition suddenly begins.
4. This critical concentration is dependent on the enzyme concentration.
5. At low purine concentrations the reaction velocity is independent of methylene blue concentration.
6. At high purine concentration the reaction velocity depends upon methylene blue concentration.
7. At low substrate concentration, the ratio of the velocities with hypoxanthine and xanthine is two to one, while at high concentrations the ratio becomes one to one.

By making two assumptions it is possible to bring these apparently disconnected facts together to form a coherent and consistent scheme. These assumptions are first, that the enzyme activates purine bases at a constant rate, i.e. one which is independent of their concentrations; and secondly, that as the purine concentrations increases it becomes increasingly difficult for the methylene blue to reach the enzyme surface.

In the light of these two assumptions we shall now consider one by one the above-mentioned experimental facts.

1. The hypoxanthine and methylene blue are in all probability adsorbed on the enzyme side by side, and there react. At low purine concentrations the enzyme is readily accessible to the methylene blue and therefore the reaction proceeds at a velocity which is determined by the rate at which the hypoxanthine is activated by the enzyme. At high purine concentrations, however, according to assumption two, it becomes difficult for the methylene blue to gain access to the enzyme surface, and the rate of methylene blue supply becomes limited. The reaction, therefore, no longer proceeds at its former rate, but with a velocity limited by the rate at which methylene blue can reach the surface of the enzyme.

2. The initial constant velocity follows from the first assumption. As long as the methylene blue has free access to the enzyme surface the reaction velocity will depend on the rate of hypoxanthine activation.

3. According to the above assumptions, as the concentration of purine increases, the methylene blue finds it increasingly difficult to reach the enzyme surface, and a stage is finally reached when its rate of supply to the enzyme is equal to the rate at which hypoxanthine is activated. Any further increase in purine concentration will result in a slowing of the rate of methylene blue

supply below this value, and the reaction velocity will then be limited by this rate of supply and not by the rate of hypoxanthine activation. Any increase of purine concentration beyond that at which the two rates are equal will cause inhibition.

4. The fact that the critical concentration depends on the enzyme concentration indicates that much of the purine in solution is associated with the enzyme, because adding more enzyme causes the purine to be distributed over a larger enzyme surface, and hence so great a resistance is not offered to the passage of methylene blue. Otherwise the addition of more enzyme would not decrease the inhibitory effect of the purine.

5. Since at low purine concentration the reaction velocity depends on the rate of hypoxanthine activation and the methylene blue has free access to the enzyme, the concentration of methylene blue will not affect the reaction velocity.

6. Since at high purine concentrations the access of methylene blue to the enzyme is limited, the reaction velocity will depend upon the rate of access, and it is to be expected that this will depend on methylene blue concentration.

7. When the velocity is limited only by the constant rate of substrate activation which we assume to be the same for hypoxanthine and xanthine, hypoxanthine will reduce methylene blue at twice the rate that xanthine does, since it uses two molecules of methylene blue for its conversion to uric acid whereas xanthine uses but one. Hence the ratio of their rates of reduction of methylene blue is two to one. When, however, inhibition occurs and the velocity comes to depend upon the rate at which methylene blue reaches the enzyme, this rate will be the same for both xanthine and hypoxanthine, and therefore their rates of reduction will be one to one. That the curves show a slight crossing indicates that hypoxanthine is slightly more effective than is xanthine in causing inhibition.

At the present stage of knowledge of enzyme reactions it is extremely difficult to formulate any theory along physico-chemical lines which would explain either the constant rate of hypoxanthine activation by the enzyme or the blocking of the enzyme surface by purines. The temperature coefficient of the reaction, which has been found to have a value of 2·0, indicates that the activation is a true chemical process and one which is not limited by the rate of diffusion of the substrate to the surface of the enzyme. It seems impossible, however, to picture any mechanism by which the rate of activation can remain independent of the substrate concentration. In view of the fundamental importance of obtaining a clearer conception of the nature of activation by enzymes it seems desirable that further work should be done along this line.

In regard to the second of these assumptions, namely that the surface of the enzyme is rendered less accessible to methylene blue by purine in the higher concentrations, there seems to be no doubt from experimental observations that this is actually what occurs, but we are unable to state without further investigation by what precise mechanism such an effect is brought about.

Identity of Enzymes.

The question was raised by Morgan, Stewart and Hopkins as to the possibility of the xanthine oxidase being identical with the Schardinger enzyme. As they pointed out, it seems at first sight improbable that an enzyme showing such marked specificity in the purine group itself should extend its activity to substances differing so widely from purines as aldehyde. They also used as an argument against the identity of the two enzymes the fact that the hypoxanthine reaction proceeds with a much higher velocity than does the aldehyde reaction. This, however, seems to us not to be valid evidence, as several cases are known where one enzyme is capable of bringing about different reactions at widely different rates. We have made an observation which might at first seem to be evidence against the identity of the two enzymes. A solution of enzyme preparation at p_H 4·1 to 4·3 is completely inactive with hypoxanthine, but reacts with aldehyde fairly rapidly. Although this seems to show a destruction of one enzyme and not the other, it has already been shown in previous experiments that the loss of activity for hypoxanthine is merely a p_H effect on the reaction. It seems to us probable that even if the reactions were brought about by the same enzyme they might not be affected by p_H in exactly the same way.

The evidence against the identity of the two enzymes seems therefore to be inconclusive. We have, on the other hand, accumulated a large number of observations which suggest the probability of the enzymes being identical. We do not, however, feel that at present the evidence is sufficient to justify a positive statement to this effect. In the first place we have been unable by any means to effect a separation of the enzymes. Both are completely precipitated from milk with the caseinogen. Neither is removed by the ether extraction. The aldehyde activity of a well-preserved preparation is retained as long as is that with hypoxanthine, but both are very slowly lost as the dry preparation stands in air. As has been mentioned before, the enzyme in a solution exposed to air loses both its aldehyde and hypoxanthine activity. Both enzymes are removed by filter-paper, and are also destroyed in the same degree by heating for a short time.

In the second place, a comparison of a large number of preparations from different milks shows a striking parallelism between the two activities. In no case have we been able to obtain two preparations whose activities varied inversely, one going quicker with hypoxanthine than with aldehyde, the other going quicker with aldehyde. Morgan, Stewart and Hopkins stated that in milk the two activities do not always run parallel with each other. This could be explained by the fact which we have found that fat accelerates the reaction with hypoxanthine but not that with aldehyde. Even if the two processes were brought about by the same enzyme, if the milks contain varying amounts of fat the activities would not be parallel. In our preparation, of course, the fat has been removed.

As stated above, the p_H curves of the two are very similar, but this is not necessarily evidence for their identity. The substrate concentration curve with aldehyde is not of the same form as that given with purines, but this is not to be expected, as the inhibition in all cases so far examined has been found to be a specific property of purines. The aldehyde concentration curve is given in Fig. 10. It can be seen that the velocity is directly proportional to the concentration of aldehyde. This does not necessarily show a difference in enzymes, but merely that the aldehyde does not block the surface of the enzyme as do purines. The purine inhibitory effects, however, are the same for both enzymes, if indeed there be two. The inhibitory effect of uric acid on hypoxanthine has already been shown, and if the enzymes were identical a similar inhibition by purines would be expected in the case of aldehyde. Such inhibition was actually found to occur, as shown in Fig. 11.

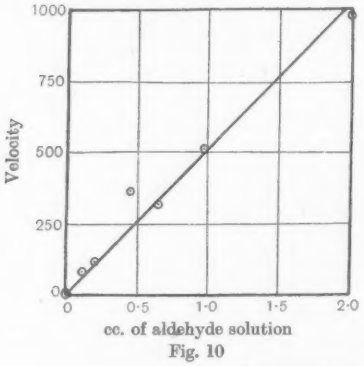

Fig. 10

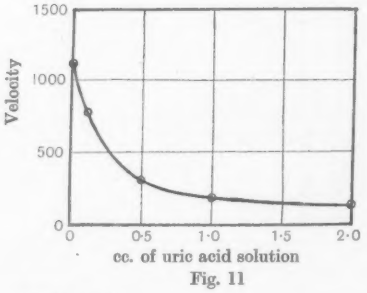

Fig. 11

Fig. 10. *Aldehyde concentration curve.*

Each tube contained: 5·0 cc. enzyme solution (in buffer); 0·5 cc. methylene blue; x cc. 10 % aldehyde (neutral); $(2-x)$ cc. water.

Fig. 11. *Inhibition of aldehyde reaction by uric acid.*

Each tube contained: 5·0 cc. enzyme solution (in buffer); 0·5 cc. methylene blue; 0·1 cc. 10 % acetaldehyde (neutral); x cc. uric acid (5 mg. per cc.); $(2-x)$ cc. water

Caffeine, however, as was the case with hypoxanthine, showed no effect. $N/100$ sodium fluoride and cyanide showed a very slight inhibitory action on both hypoxanthine and aldehyde. In the presence of either alcohol or acetone the activity of both enzymes was markedly and proportionately decreased. In view of this remarkable similarity in behaviour we feel that the balance of evidence is in favour of the two enzymes being identical.

We now turned our attention to the possibility of the oxidation of adenine being due to the same enzyme. In the case of adenine there are two reactions which might occur. The adenine may be deaminated first by adenase, as supposed by Morgan, Stewart and Hopkins, and the resulting hypoxanthine oxidised by xanthine oxidase; or it is possible that xanthine oxidase might oxidise adenine directly, without the adenine undergoing deamination, in

which case it would yield 6-amino-2-8-dioxypurine. Such an oxidation is known to occur in the animal body. If adenase is present it must be in very small quantities, since the reaction with adenine is very slow. Therefore the reaction velocity will be controlled by the speed at which the adenine is deaminated, the hypoxanthine being oxidised as fast as formed.

Since adenase is a deaminase and the xanthine enzyme an oxidase, one would not expect the two enzymes to be very similar in behaviour, e.g. with respect to p_H. As has been stated, however, the p_H curves for hypoxanthine and adenine are very similar, exhibiting the break at p_H 9·0. The substrate concentration curve for adenine, when carried out with a range of adenine concentrations similar to that used for hypoxanthine, is flat, that is, the velocity is independent of the adenine concentration. When, however, a more concentrated adenine solution is used, the velocity is found to decrease with increasing adenine concentration, and the curve resembles that obtained with hypoxanthine and xanthine. The two curves for adenine are shown in Figs. 12 and 13.

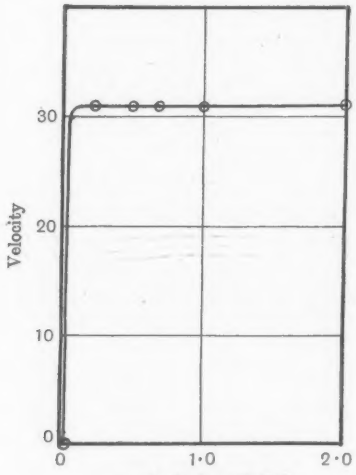

Fig. 12

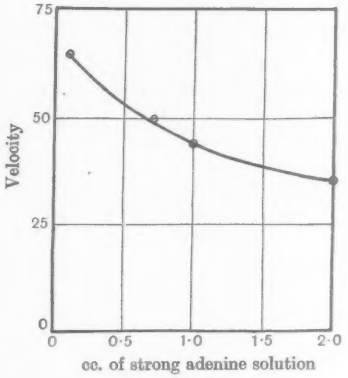

Fig. 13

Fig. 12. *Adenine concentration curve.*

Each tube contained: 5·0 cc. enzyme solution (in buffer); 0·5 cc. methylene blue: x cc. adenine (2 mg. per cc.); $(2-x)$ cc. water.

Fig. 13. *Adenine concentration curve (higher concentration).*

Each tube contained: 5·0 cc. enzyme solution (in buffer); 0·5 cc. methylene blue; x cc. adenine (5 mg. per cc.); $(2-x)$ cc. water.

When using adenine as a substrate it was necessary to use very strong enzyme solutions, since otherwise the reduction times were too long for convenient observation. This explains why the critical adenine concentration is

so much higher in these experiments than the critical hypoxanthine concentration. The fact that these curves are of exactly the same type as the hypoxanthine curves is strong evidence in favour of the view that the same enzyme brings about both reactions. Moreover, the effect of inhibiting substances, uric acid, fluoride and cyanide, is the same for adenine as for hypoxanthine, while caffeine again has no effect. The parallelism in activity of various preparations with respect to hypoxanthine and adenine has been observed in every preparation used.

If adenine were directly oxidised, aminodioxypurine would be formed instead of uric acid. This has not yet been settled experimentally. It seems, therefore, at the moment that the evidence in either direction is inconclusive, but with the balance of evidence in favour of identity.

Summary.

1. An investigation of the dynamics of the xanthine oxidase has been carried out by means of the methylene blue technique.

2. The reaction velocity is not affected by p_H between p_H 5·5 and 9. Above p_H 9 and below p_H 4 the enzyme is destroyed. Between p_H 4 and 5 the reaction velocity is very small but no destruction occurs.

3. The reaction velocity is directly proportional to the enzyme concentration, but is independent of methylene blue concentration provided this is not very small.

4. When the concentration of purine base is increased beyond a certain critical concentration the reaction velocity is decreased. Below this critical concentration the reaction velocity is independent of purine base concentration.

5. Upon addition of purines (e.g. uric acid), other than the substrate the reaction velocity is similarly decreased.

6. The critical concentration of purine base at which inhibition begins increases with increasing enzyme concentration.

7. Below the critical substrate concentration, hypoxanthine reduces methylene blue twice as fast as does xanthine, but as inhibition begins the rates approach one another and become equal when inhibition is complete.

8. When inhibition is complete the reaction velocity is dependent upon methylene blue concentration.

9. The bearing of these facts on the mechanism of the oxidase system is discussed.

References.

Dixon and Tunnicliffe (1923). *Proc. Roy. Soc. Lond.* **94** B, 266.
Morgan, Stewart and Hopkins (1922). *Proc. Roy. Soc. Lond.* **94** B, 109.
Ohlsson (1921). *Skand. Arch. Physiol.* **41**, 77.
Widmark (1921). *Skand. Arch. Physiol.* **41**, 200.

CXXXII. STUDIES ON XANTHINE OXIDASE.

III. THE REDUCTION OF NITRATES.

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From the Biochemical Laboratory, Cambridge University.

(*Received July 31st, 1924.*)

In a recently published paper, Haas and Hill [1923] described the property possessed by milk of reducing nitrates in presence of aldehydes as "activators." This observation had previously been made by Bach [1911]. To the substance present in the milk which causes this reduction they gave the name "atite," and they regarded the "atite" as the actual "oxidisable substance," or, in other words, reducing agent, the aldehyde acting as an accelerator or apparently a catalyst.

It is well known that milk contains an enzyme, Schardinger's enzyme, capable of oxidising aldehydes in presence of such substances as methylene blue which themselves become reduced in the process. It occurred to us that the aldehyde in the system described by Haas and Hill might really be the reducing substance and not the catalyst. In this case the Schardinger enzyme would be the catalyst and the nitrate would simply be taking the place of the methylene blue, so that the term "atite" would merely designate the oxidising enzymes present in milk.

Haas and Hill gave reasons for believing that "atite" was not identical with the Schardinger enzyme. They stated that on heating milk to certain temperatures they could destroy the Schardinger enzyme without destroying "atite." They do not, however, state the amount of methylene blue used in this particular experiment, but in other similar experiments where quantities are given they use twenty times the usual amount employed in the methylene blue technique. Moreover, they give no actual reduction times and merely indicate their results as positive or negative. Thus a solution containing a small amount of enzyme would be considered by them to give a negative result, as the reduction time with so large an amount of methylene blue might easily be several days. On the other hand, the Griess-Ilosvay test for nitrites is an extremely delicate one so that very small amounts of enzyme would be detected. It does not therefore seem proved by their results that they actually effected a separation, particularly as they state that when the Schardinger reaction was first observed to be negative, the nitrite reaction was also very weak. We have repeated their experiments using smaller quantities of methylene blue and have been unable to effect a separation of the two activities. Haas and Hill

also stated that the nitrate-reducing agent of the potato was not identical with the Schardinger enzyme, but we have been led to believe that the "atite" of milk is different from the "atite" of the potato, as we have found that the two are "activated" by different substances. This will be explained later on in the paper. Since our evidence shows that the systems in the potato and milk are different we feel that arguments based on the properties of one system are inconclusive when applied to the other.

On the other hand, there are a number of facts which seem to point to the identity of "atite" with the xanthine and aldehyde oxidases of milk, which, as we have shown in the second paper of this series, are probably one and the same. Wherever the oxidase has been found we have found also the power to reduce nitrates in the presence of aldehyde. In the first paper of this series we gave a method for the preparation of the milk oxidases, i.e. the xanthine and aldehyde oxidase. This active caseinogen preparation was found to reduce nitrates as well as milk did in the presence of aldehyde. On clotting milk with rennin, both the oxidase and "atite" were found in the whey. Filter-paper removes a large proportion of the oxidase as shown in the former papers of this series, and the "atite" is similarly removed by this procedure.

If the hypothesis that the aldehyde is merely the reducing substance is true, it would be expected that hypoxanthine and xanthine could replace the aldehyde in the system. This was indeed found to be the case. Both milk and our caseinogen preparation in the presence of hypoxanthine actively reduce nitrate to nitrite. In fact all those substances which in the previous papers were found to be oxidised by the milk oxidase were able to bring about the reduction of nitrate. Furthermore, the amount of nitrite produced in a given time in presence of different substances was exactly parallel to the rate of reduction of methylene blue by these substances.

Since Haas and Hill have shown that milk contains an agent which will oxidise nitrites in the presence of oxygen, these experiments were carried out in vacuum tubes. The procedure employed was the same as that described in the previous papers. After standing in a thermostat for a definite time the tubes were opened and the Griess-Ilosvay reagent added immediately. The following table gives a typical experiment.

Each tube contained 5 cc. enzyme solution + 1 cc. 5 % $NaNO_3$ or 0·5 cc. methylene blue and 0·5 cc. water + 0·1 cc. of substance given in column 1. The evacuated nitrate tubes were heated ½ hour in a water-bath at 37°.

Substance	Nitrite test	Reduction time
Control	−	∞
Hypoxanthine (2 mg. per cc.)	+ + + + +	2′
Xanthine (2 mg. per cc.)	+ + +	4′
Aldehyde (10 %)	+	58′
Adenine (2 mg. per cc.)	+	50′
Uric acid (20 mg. per cc.)	−	∞
Guanine (2 mg. per cc.)	−	∞
Caffeine (20 mg. per cc.)	−	∞

If the nitrate is merely replacing the methylene blue in this system, it would be expected that the substances that inhibit methylene blue reduction

would also inhibit nitrite formation. The following table shows the parallelism in inhibitory effects between the reaction with methylene blue and that with nitrate.

Each tube contained 5 cc. enzyme solution + 1 cc. 5 % $NaNO_3$ or 0·5 cc. methylene blue + 0·1 cc. Hx (2 mg. per cc.) + 0·5 cc. of substance in column 1.

Inhibiting substance	Nitrate test	Reduction time
Control	+ + + +	3′
N/10 KCN	+	7′
1 % NaF	+ + +	4′
Guanine (2 mg. per cc.)	+	15′
Caffeine (20 mg. per cc.)	+ + + +	3′
Uric acid (20 mg. per cc.)	+	15′

As has been shown in the previous paper, the oxidase begins to undergo destruction at p_H 9·3, and on adjusting the p_H of the solution to this value the nitrate-reducing power was similarly diminished.

It is of interest that the same inhibition by purines that was observed in the previous paper occurs also with nitrates, since this indicates that this is not a specific property of methylene blue. We have observed the same effect as before with high concentrations of hypoxanthine. The reduction of nitrate proceeds much more rapidly with a small than with a large concentration of hypoxanthine.

This very striking correspondence seems definitely to establish the view expressed above that the nitrate simply serves as an oxidising agent, replacing methylene blue, and that the "atite" of milk is merely another name for the oxidising enzymes of milk. The "atite" of the potato we have found to be activated by formaldehyde and acetaldehyde but not by the purine bases, in this respect contrasting sharply with the milk "atite." We think this is evidence that the two "atites" are of different natures.

It will be seen that the reduction of nitrate to nitrite gives us another technique for the investigation of oxidising enzymes and tissue respiration generally, and can be employed as an alternative to the methylene blue technique of Thunberg [1920] and others, and the dinitrobenzene technique of Lipschitz and Gottschalk [1921]. The method can easily be made accurately quantitative by the use of the colorimetric estimation method with metaphenylenediamine; or qualitative results can be quickly obtained by means of the Griess-Ilosvay reagent.

Summary.

1. Milk or the preparation of xanthine oxidase will reduce nitrates to nitrites in presence of hypoxanthine, xanthine, adenine or aldehyde.

2. The nitrate takes the place of the methylene blue in the system described in the previous papers.

3. The rate of reduction of nitrate is proportional to the rate of reduction of methylene blue under various conditions.

4. The inhibition effects by purines previously observed with methylene blue also occur with nitrates.

5. The "atite" of Haas and Hill is identical with the oxidase or oxidases in milk.

We wish to express our indebtedness to Professor F. G. Hopkins for his continual interest during this work and for much helpful advice.

REFERENCES.

Bach (1911). *Biochem. Z.* **33**, 282.
Haas and Hill (1923). *Biochem. J.* **17**, 671.
Lipschitz and Gottschalk (1921). *Pflüger's Arch.* **191**, 1.
Thunberg (1920). *Skand. Arch. Physiol.* **40**, 1.

CXXXIII. THE ACID-BASE EXCHANGE IN MAMMALIAN VOLUNTARY MUSCLE.

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Introduction.

There are present within the muscle inorganic and organic systems which can part with their base to neutralise the acids produced during activity or in the tissue after death. There have been many investigations within recent years which deal with the acid production in muscle, the extremely important researches of Fletcher and Hopkins [1906] having been followed by those of Embden and his school [1912; 1914, 1, 2; 1921], Parnas and Laska-Mintz [1921], Meyerhof [1922] and many others and the results so obtained have been of great value in the study of the energy changes in muscle [A. V. Hill and Meyerhof, 1923]. It is necessary in the study of the heat production in muscle to obtain as much information as possible regarding the carriers of base which can part with their alkali within the zone of reaction change near the neutral point. The amount of base which can be obtained between a more alkaline and a less alkaline point by such an acid as lactic acid depends upon the absolute concentrations and the dissociation constants of the acid groups which hold the disposable base. As Meyerhof has pointed out, the exothermic changes associated with the removal of base from colloidal systems and from the amino acids are of a high order, dependent upon the heat set free in the passage from the dissociated salt form to the undissociated colloidal acid or amino acid form. The part played by the colloidal protein systems in these changes within such a reaction zone as from p_H 7·5 to p_H 6·57 will depend upon the amount of base which can be secured from such systems by lactic acid or other acids. If the muscle proteins have an acid dissociation constant of the value $10^{-5\cdot7}$ as Meyerhof [1922] calculates from the K_b of horse serum albumin given by Pauli [1920] and the isoelectric point of serum albumin [Michaelis and Davidsohn, 1911], then undoubtedly the amount of acid which may be produced between these two points could not secure the necessary amount of base from such a system alone. Thus, even if we take the concentration of the carboxyl groups in the proteins of expressed muscle juice at so high a figure as 0·05 molar, the amount of base which could be secured between these two reaction points would only be approximately 9·5 millimols[1], because at the

[1] All concentrations referred to are per litre.

slightly alkaline reaction of p_H 7·5, the ratio of undissociated acid to colloidal anion would be $\frac{\cdot 0015}{\cdot 0485}$, and at the slightly acid reaction of p_H 6·57, the ratio would be $\frac{\cdot 011}{\cdot 039}$. Between these two points, therefore, 9·5 millimols base are obtainable for the neutralisation of an approximately equal number of millimols lactic acid, less than 1 % lactic acid being free even at p_H 6. As there may be a much greater increase in lactic acid, apart from other acid production, between these two points, it is evident that under these conditions base must be secured from some other source. As even at the more acid reaction the muscle juice is on the alkaline side of the iso-electric point of serum albumin ($2 \cdot 10^{-5}$) and therefore probably also of that of the muscle proteins, we may regard such a system as a base-carrying one. Naturally acids so weak as the amino acids (K_a 10^{-10}) and creatine cannot carry base on the acid side of neutrality. There are at least four possible base-carrying systems which might be considered, namely, bicarbonates, inorganic phosphates, the organic forerunner of the inorganic phosphates ("lactacidogen" of Embden) and the proteins. As regards the pre-formed bicarbonate the concentration even in the fresh muscle is extremely small, according to Meyerhof 0·005 molar, and in the expressed juice with which this paper deals the amount present is still less, so that attention will be directed to the remaining three systems. The concentration of the pre-formed phosphate varies from about 0·015–0·03 molar, and the lactacidogen forerunner is present also in variable but somewhat similar concentration. There are no data at present available regarding the molar concentration of the protein carboxyl groups in muscle juice.

Experimental.

The investigations dealt with in the following paper are concerned with the expressed juice of muscle. It was fully recognised that even with the greatest care in preparation, there will always be, in the case of expressed juice, a distinct acid production during the processes of extraction. Information of an important kind may, however, be obtained by comparing the reaction swing with the lactic and phosphoric acid increments on passing from a cooled to a warmed juice, even if the reaction of the former be distinctly more acid than that of the living tissue. In addition, it was regarded of importance to study the ultrafiltrates of the juice from frozen muscle and from the same juice after warming, in order to determine the part played by the colloidal and the non-colloidal constituents. Special attention was directed to the expressed juice of horse and dog muscle. The methods adopted for the preparation of the juice and the ultrafiltrates were as follows.

The animals were bled and, in the case of the dog, the blood was thoroughly washed out from the vessels. The muscles were then sprayed with ethyl chloride, carefully removed and frozen by being placed at once in vessels

immersed in freshly dissolved ammonium nitrate. In some cases liquid air was used, but in certain respects the after treatment of the hard frozen muscle was found to be troublesome and unsatisfactory. After freezing in the ammonium nitrate solution the muscle was rapidly minced, mixed with sand and the juice expressed in a porcelain filter-press. The juice was kept in cooled Dewar flasks until required for analysis.

The reaction of the cold juice was determined electrometrically and electrometric titration with known additions of acid or alkali carried out in order to determine the buffer value of the juice.

The pre-formed inorganic phosphate was determined by Embden's gravimetric method [1921] after removal of the protein by trichloroacetic acid. The lactic acid was determined by Meyerhof's method [1922] and the total nitrogen by Kjeldahl's method. The latter was determined simply to discover whether the concentration of the juice varied in different specimens. Another portion of the muscle juice was warmed for two hours at 40° and the reaction and buffer capacity determined in the same way and at the same temperature as in the cooled juice. The same analyses were made in the case of this warmed juice. The ultrafiltrates from the cold and warm juice were obtained in the following way. The juice was poured from the Dewar flask into a bottle, kept in melting ice, which was connected up to a gas cylinder with a tyre foot pump and with a manometer attached to a T-piece between cylinder and bottle. The exit tube from the bottle was attached to the filter-press (Walpole type) carrying two celloidin filters. Before filling the bottle with the juice the water held by the celloidin plates was driven out under pressure. About 100 cc. juice were used for filtration and the last 10–15 cc. of the first 50 cc. of the filtrate were taken for the reaction determinations and analyses. The pressure was then lowered, and the juice between the plates sucked back into the bottle. The bottle was then kept in a bath at 40° for 2 hours, and the filter-press clamping plates were also warmed. The pressure was again raised and the second portion of the ultrafiltrate from the warm juice taken for the necessary determinations.

Thus the cold and warm juice and their respective ultrafiltrates were examined and, upon the data so obtained, the study of the acid base changes produced by warming was based.

Horse Muscle.

In the first case the juice alone will be considered.

Exp. 1. *Analyses.* The cold and the warmed juice contained 1·132 % nitrogen.

The inorganic phosphate (precipitable) and the lactic acid values in the deproteinised specimens were as follows:

	H_3PO_4 (molar)	Lactic acid (molar)
Cold	·026	·026
Warmed	·041	·060

The increment in lactic acid was therefore rather more than double that in the inorganic phosphate, so that if these had a common source such as a hexose-phosphoric acid ester [see Harden, 1923], the forerunner would be of the nature of a hexose-monophosphoric acid.

Reaction determinations. 1. *Juice expressed from frozen muscle.* Reaction (18°) p_H 6·77. At this reaction the ratio of K_2HPO_4 to KH_2PO_4 (from electrometric determinations; in the sequel simply termed the ratio) corresponds to per mol phosphoric acid $\frac{455·2}{544·8}$ in millimols (mmols.).

5 cc. of this juice were taken and 0·2 cc. of 0·3*N* KOH added (= 12 mmols. base per litre), and the reaction was again determined at 18° when p_H was found to be 7·44. At this reaction the ratio would be $\frac{804}{196}$, an increase of 348·8 mmols. per mol phosphoric acid, and therefore the 12 mmols. actually added correspond to 0·0344*M* phosphoric acid. In other words, a 0·0344 molar phosphate solution of p_H 6·77 would show a reaction swing to p_H 7·44 on addition of 12 mmols. base per litre, and therefore represents the buffer value of the juice between these two reactions. On reversing the process, the addition of 12 mmols. acid to the juice at p_H 7·44 changed the reaction to the original 6·77, so that the buffer value had not altered during the period between the two determinations.

2. *The same juice after warming for 2 hours at 40°.* The reaction was now p_H 6·18 (at 18°), and it required 12 mmols. base to shift the reaction to p_H 6·72 (approximately that of the original cold juice). This corresponds to a buffer value of 0·046*M* phosphate. To determine the buffer capacity on the alkaline side of p_H 6·72, 18 mmols. more base were added when the reaction was found to have shifted to p_H 7·36, equivalent in buffer capacity to 0·052*M* phosphate.

It is evident, therefore, that the buffer capacities of the cold and warmed juice are higher than correspond to their actual phosphate contents.

Knowing the inorganic phosphate concentrations, and the reactions of the cold and warmed juice, the total base content of the phosphate systems at p_H 6·77 and at p_H 6·18 can be calculated. At p_H 6·77, the ratio in 0·026*M* phosphate is $\frac{·0118}{·0142}$ which therefore gives for the cold juice a total base content in its phosphate system of 37·8 mmols.

At p_H 6·18, the ratio in 0·041*M* phosphate is $\frac{·0068}{·0342}$, which gives for the warmed juice a total base content of 47·8 mmols. or an increase of 10 mmols. base. What is the source of this base? If the increase in phosphoric acid be regarded as derived by hydrolysis from a forerunner such as hexosemonophosphoric acid then the alkaline salt of this acid may be regarded as holding the same amount of base as phosphoric acid at the same reaction, for example, two base equivalents for each atom of phosphorus at the phenolphthalein change point, as shown in the following equation:

$$C_6H_{11}O_5(PO_4K_2) + H_2O = C_6H_{12}O_6 + K_2HPO_4.$$

From the hexose so set free two molecules lactic acid may be derived, one

molecule of which, in the absence of any other source of base, would change the dibasic to the monobasic salt, giving a reaction shift of approximately p_H 9 to p_H 4[1].

As the increment in precipitable phosphate in the warmed juice is derived from a body existent in the cold juice, in all probability of the nature of a hexose-phosphate, the total molar concentration of the cold juice may be regarded as 0·041 (0·026 precipitable and 0·015 hydrolysable) and this system may possibly contain the same amount of base as a phosphate solution at the same reaction. At p_H 6·77, the cold juice considered as 0·041M phosphate solution should contain the maximal amount of base derivable from this source, and this value can be determined from the ratio of dibasic to monobasic at this reaction, namely $\frac{\cdot 0186}{\cdot 0224}$. At p_H 6·18 the ratio is $\frac{\cdot 0068}{\cdot 0342}$, so that, on passing from the less to the more acid, 11·8 mmols. base have been removed. The amount of lactic acid actually produced between the two reaction points was 34 mmols. (33·8 of which would secure base at p_H 6·18). This increment in lactic acid would therefore require 22 mmols. base from some other source for its neutralisation. If the original substance were the salt of a diphosphoric acid ester, then lactic acid and phosphoric acid would appear in equimolecular proportions and of the 34 mmols. lactic acid actually produced, only 15 (equal to the H_3PO_4 increment) would come from this source and 19 mmols. from some other source along with their base equivalent. In both cases the "lact-acidogen" system is unable to furnish the amount of base which must have been secured by the lactic acid produced on warming the juice, when the reaction shifted from 6·77 to 6·18. That base has actually been derived from acid groups other than the phosphoric is evident from the capacity of the warmed juice to take up more base between the reactions of the warmed and cold specimens than would be the case with a pure solution containing the same amount of phosphate. One must always bear in mind that in addition to the acid determined (lactic), other acids such as volatile fatty acids with K_a of the value 10^{-5} may be produced and these will contribute to the increase in the buffer capacity of the juice owing to their removal of base, for example, from the proteins. When alkali is added, therefore, to the warmed juice, part of this is required to satisfy the base requirements of the colloidal acid groups as well as of the phosphoric in the phosphate system.

Exp. 2. (Horse Muscle.) In this case both the juice and the ultrafiltrates will be considered.

The nitrogen percentage of the cold and warmed juice was 1·221.

Analyses	Inorganic phosphate (molar)	Lactic acid (molar)
(*a*) Cold juice	·0228	·0254
(*b*) Ultrafiltrate of cold juice	·0240	·0252
(*c*) Warmed juice	·0335	·0389
(*d*) Ultrafiltrate of warmed juice	·0340	·0386

[1] The references to this organic forerunner are based upon the studies of the phosphate mechanisms in yeast (Harden), our knowledge of the true nature of this precursor in muscle being very incomplete. Further investigation of this subject is absolutely necessary.

For 10 mmols. increase in phosphoric acid, there is therefore approximately 13 mmols. lactic acid increment, so that the process of warming has given rise approximately to an equimolecular increase in these constituents. The phosphate determinations are undoubtedly more accurate than those of lactic acid.

Reaction Determinations (Fig. 1).

(*a*) Juice (frozen muscle).

1. p_H (18°) 6·77, ratio $= \frac{455·2}{544·8}$.

2. + 6 mmols. base, p_H 6·98, ratio $= \frac{580·4}{419·6}$.

Buffer capacity (on alkaline side) = 0·047*M* phosphate.

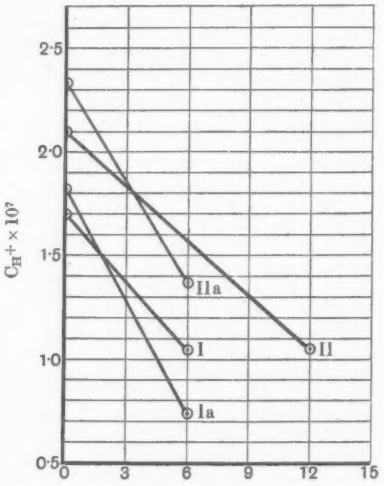

Fig. 1 (Exp. 2). I, Cold muscle juice; Ia, Ultrafiltrate of same; II, Heated juice; IIa, Ultrafiltrate of same (Horse).

To the juice at p_H 6·98, 12 mmols. acid were added, and the reaction shifted to p_H 6·47, ratio $\frac{288·4}{711·6}$.

Buffer capacity (on acid side) = 0·041*M* phosphate.

(*b*) Ultrafiltrate of cold juice.

1. p_H (18°) 6·74, ratio $\frac{438}{562}$.

2. + 6 mmols. base p_H 7·13, ratio $\frac{662·4}{337·6}$.

Buffer capacity (alkaline side) = 0·026*M* phosphate.

To the juice at p_H 7·13, 12 mmols. acid were added, and the reaction shifted to p_H 6·35, ratio $\frac{232}{768}$.

Buffer capacity (acid side) = 0·027M phosphate.

(*c*) Warmed juice.

1. p_H (18°) 6·56.
2. + 6 mmols. base, 6·68.
3. + 18 „ „ 6·98.
4. + 24 „ „ 7·11.

Average buffer capacity (on alkaline side) = 0·076M phosphate.

(The ratios of dibasic to monobasic will now be omitted.)

(*d*) Ultrafiltrate of warmed juice.

1. p_H (18°) 6·63.
2. + 6 mmols. base p_H 6·86.

Buffer capacity (on alkaline side) = 0·044M phosphate.

To the ultrafiltrate at p_H 6·86, 12 mmols. acid were added, and reaction shifted to p_H 6·39; buffer capacity (acid side) = 0·045M phosphate, and on addition of 12 mmols. base to this solution, the reaction was reversed to 6·86.

The average buffer capacities and the actual phosphate concentrations may then be stated as follows:

	Buffer capacity (molar phosphate)	Phosphate (determined) (molar)
Cold juice	·0440	·0228
Ultrafiltrate	·0265	·0240
Warmed juice	·0760	·0335
Ultrafiltrate	·0445	·0340

It is evident that warming muscle juice increases the buffer capacity of the specimen just as it raises the precipitable phosphate content, but in all cases the buffer capacity is higher than the contained phosphate. There is, however, a much closer relationship between the buffer capacity and the phosphate content in the case of the ultrafiltrates than is the case with the original juice. The buffer value of the ultrafiltrate of the cold juice is 10 % higher than that of the contained phosphate, and in the filtrate of the warmed specimen 30 % higher, while the cold juice has a buffer value 93 % higher and the warmed 126 % greater than their respective phosphate contents. That is to say, on testing the buffer capacity of the juice by the addition of alkali, acid groups, other than the phosphoric, are present which soak up the added base between the reaction points determined. These acid groups which have been deprived of base by the acid production in muscle juice belong mainly to the colloidal constituents and therefore do not pass into the ultrafiltrates, although, especially in the case of the filtrate from the warmed juice, there are non-colloidal acid groups in addition to phosphoric which secure a small amount of base between the two reaction points.

The base contents of the inorganic phosphate systems of the cold and warmed juice will now be considered. The cold juice at p_H 6·77 with 0·0228M

H_3PO_4 had the ratio $\frac{\cdot0103}{\cdot0125}$ and after warming at p_H 6·56 with 0·0335M phosphate, $\frac{\cdot0108}{\cdot0227}$. The increase in the base content of the latter system over the former was 11·2 mmols. As in the preceding experiment, the cold juice may be regarded as originally containing 0·0335M phosphate (inorganic + hydrolysable) which at p_H 6·77 would give the ratio $\frac{\cdot0152}{\cdot0183}$ and for the warmed juice at p_H 6·56 $\frac{\cdot0108}{\cdot0227}$. The amount of base derivable from this system between these two reactions is then 4·4 mmols. which would neutralise slightly under that value of lactic acid. The increment, however, in lactic acid between these two points was found to be 13 mmols., so that approximately 9 mmols. acid would require to secure base from some other source, raising as a result the buffer capacity on that side above the value dependent upon the phosphate present. The colloidal acid groups deprived of, approximately, 9 mmols. base to neutralise this excess of lactic acid, which could not be neutralised by the base of the phosphate systems, would then require to take up this base equivalent in order to bring the reaction back to that of the cold juice. These values can merely be regarded as approximately true, in the first place, because lactic acid determinations are never sufficiently accurate, and, in the second place, other acids may be set free, which are not estimated, and yet which may secure base under these conditions.

In the preceding experiments there was some degree of proportionality between the phosphoric and lactic acid increments produced by heating the juice, agreeing more or less closely with the two types described by Embden, one in which the forerunner is a hexosemonophosphate, and the other, a hexosediphosphate.

In some cases, however, there may be a great departure from either of these types, in one the phosphoric may increase without the lactic acid showing even an equivalent rise, or, in the other, the lactic acid may rise without a proportional rise in the phosphoric acid.

In the former case, as will be seen from the next experiment, the amount of base which can be taken up between two reaction points does not rise so much on heating as in the preceding experiments, showing that the phosphate arises from a base-carrying source which can supply the necessary amount of base for the inorganic phosphate derived from it. An example of this kind will now be given.

Exp. 3. (Horse muscle.) The original reactions and the reaction swings on addition of base will be given in condensed tabular form, the buffer capacity being determined between the original reactions of the various specimens and the reactions obtained after the addition of the maximal amount of base.

Analyses of these specimens.

	Inorganic phosphate H_3PO_4 (molar)	Lactic acid (molar)
Juice (cold)	·0320	·031
Ultrafiltrate	·0222	·0244
Juice (warmed)	·0590	·039
Ultrafiltrate	·0450	·029

Reactions (Fig. 2).

	Original p_H	p_H after addition of mmols. base 3	6	9	12	18	Buffer capacity (molar phosphate)
Juice (cold)	6·75	6·85	6·92	7·04	—	—	·0530
Ultrafiltrate of cold juice	6·75	6·97	7·23	—	—	—	·0222
Juice (warmed)	6·57	—	6·74	—	6·93	7·10	·059
Ultrafiltrate of warmed juice	6·57	—	6·85	—	7·06	7·28	·0455

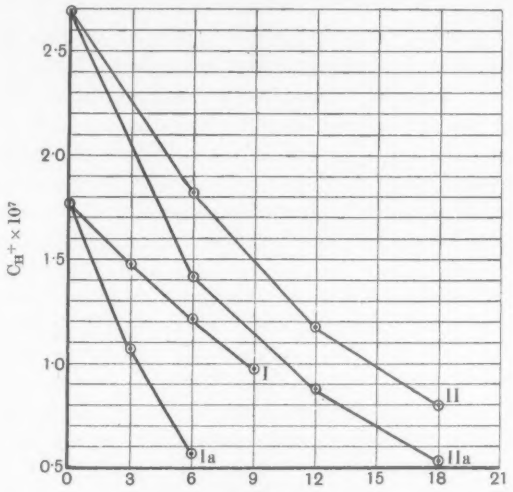

Fig. 2 (Exp. 3). I, Cold muscle juice; Ia, Ultrafiltrate of same; II, Heated juice; IIa, Ultrafiltrate of same (Horse).

There is a marked disparity between the juice specimens and their ultrafiltrates as regards their phosphoric and lactic acid contents, but in both there is the same evidence of the lactic acid increment being much below that of the phosphoric acid. The ultrafiltrates of the cold and of the heated juice and of the warmed juice before filtration possess buffer capacities which are evidently due to the phosphate system present in each. Another specimen of the same heated juice which was examined had a buffer capacity of 0·060*M* phosphate. The buffer capacity of the cold juice is much higher than its phosphate content and this is probably due to the initial high value of the lactic acid in this juice which had already deprived colloidal carboxyl groups of their base. The very slight increase in lactic acid which occurred on warming evidently secured the necessary base from the lactacidogen which had broken down without raising the buffer value of the heated much above that of the cold juice.

It is of interest to consider the base contents of the cold and warmed juice phosphate systems. The base content of 0·032*M* phosphoric acid in the cold juice at p_H 6·75, can be obtained from the ratio at this reaction, namely $\frac{\cdot 014}{\cdot 018}$,

while at the reaction of the warm juice, p_H 6·57, with 0·059*M* phosphoric acid the ratio would be $\frac{\cdot020}{\cdot039}$ or the large increase of 33 mmols. base. If the cold juice be regarded as containing originally 0·059*M* phosphoric acid (precipitable and hydrolysable), the ratio in terms of phosphate at p_H 6·75 would be $\frac{\cdot0261}{\cdot0329}$ or a total base content of 85·1 mmols., while the warmed juice with the same phosphate concentration, but all in the precipitable form, would be $\frac{\cdot020}{\cdot039}$ at p_H 6·57, or a total base content of 79 mmols., the loss of 6·1 mmols. from this system would be accounted for by the slight rise in lactic acid which would secure its base from this source and not from the colloidal constituents so that the buffering value of the latter should not rise as a result of heating.

The other case which requires consideration is one in which lactic acid rises without an inorganic phosphate increment, and therefore the former must be derived from some other source than "lactacidogen." This condition has been observed when the muscle has been kept sterile for some hours at room temperature, and the juice then expressed compared with that obtained from the juice of frozen muscle. Under such circumstances there is a greater swing in reaction towards the acid side than in cases where there is the usual phosphate increment. Such a case will now be examined.

Exp. 4. (Horse muscle.) One portion of the muscle was frozen immediately after removal, rapidly minced and the juice expressed, while the other portion was kept for 24 hours at room temperature (14°) in a sterile vessel.

The nitrogen percentage was 1·29.

Analyses. The juice only was examined.

	Phosphate (molar)	Lactic acid
Juice from frozen muscle	·030	·012
Juice from muscle after 24 hours at 14°	·033	·032

Reaction determinations.

1. Juice from frozen muscle.

Reaction (18°) p_H 7·18, rapidly changing to 7·03 after introduction of the juice into the hydrogen electrode titration vessel, so that the effect of addition of base was really studied in a juice of the latter reaction. On adding 12 mmols. base, the reaction shifted to p_H 6·34, equivalent to a buffer capacity slightly over 0·03*M* phosphate.

2. Juice from muscle after 24 hours at 14°.

Reaction (18°) p_H 5·76. In order to test the buffer capacity of this specimen within a somewhat similar range to that in which the cold juice was examined, alkali was gradually added until the reaction was 6·18. It was then tested in the usual way for its buffer value with the following results:

Original reaction p_H	6·18
12 mmols. base „	6·52
24 „ „ „	6·78
36 „ „ „	7·17

Buffer capacity between p_H 6·18 and 7·17 is equivalent to 0·061*M* phos-

phate. The base content of the 0·030M phosphate at p_H 7·03 is derivable from the ratio $\frac{·0187}{·0113}$ at this reaction, and that of the 0·033M phosphate at p_H 5·76 from the ratio $\frac{·0034}{·0296}$. It is evident that there is a loss instead of the usual gain, of 12·3 mmols. to neutralise part of the acids produced during the period at room temperature.

If the cold juice be taken as originally of p_H 7·18, and the total phosphate concentration (precipitable and hydrolysable) as 0·033M, then the ratio would be $\frac{·0227}{·0103}$, and in the juice after warming at p_H 5·76 $\frac{·0019}{·0311}$. Thus the system could have furnished 20·8 mmols. base, more than sufficient therefore to neutralise the lactic acid produced between the two reaction points. The juice at p_H 5·76 had a buffer capacity for base, however, far greater than corresponded to its phosphate content. Actually 48 mmols. base required to be added to the juice to bring it back to the reaction of the cold juice, which shows that in this case there was acid production over and above that of the 20 mmols. lactic acid.

It is evident that the changes which have taken place under those conditions are entirely different from those observed when muscle juice is rapidly warmed to 40°.

Dog muscle.

Attention will now be directed briefly to some preliminary experiments on the juice expressed from the voluntary muscle of the dog. In all cases the animals were bled and the blood washed thoroughly out of the vessels.

Exp. 1. The juice from frozen muscle and the same juice after warming will alone be considered.

Analyses.

	Phosphate (molar)	Lactic acid (molar)
Cold	·014	·006
Warmed	·025	·019

The increments are roughly equimolecular.

Reaction determinations.

1. Juice (from frozen muscle).

Original,	p_H 7·14
+ 3 mmols. acid,	6·76
+ 6 „ „	6·33
+ 9 „ „	5·80

Buffer capacity on acid side = 0·014M phosphate.

2. Juice after warming.

	p_H 6·69
+ 3 mmols. base	6·86
+ 6 „ „	7·01
+ 9 „ „	7·16

Buffer capacity on alkaline side = 0·035M phosphate.

The cold juice owed therefore its buffer capacity on the acid side to the inorganic phosphate present. Unfortunately, the nitrogen percentage was not determined, and so the strength of the solution as regards colloids is not known. The warmed juice, on the other hand, shows a greater buffer capacity than its contained phosphate, as tested by the addition of alkali.

Base content of the phosphate system before and after heating.

(*a*) At p_H 7·14 in the cold juice with 0·014*M* phosphate, the ratio would be $\frac{\cdot0093}{\cdot0047} = 23{\cdot}3$ mmols. base.

(*b*) At p_H 6·69, in the warm juice with 0·025*M* phosphate, the ratio would be $\frac{\cdot0103}{\cdot0147} = 35{\cdot}3$ mmols. base.

The inorganic phosphate therefore shows an increase of 12 mmols. base.

(*c*) At p_H 7·14, in the cold juice with 0·025*M* phosphate (precipitable + hydrolysable), the ratio would be $\frac{\cdot0167}{\cdot0083} = 41{\cdot}7$ mmols. base.

On warming the juice which in its original condition contained 41·7 mmols. base attached to the combined "lactacidogen"-phosphate system, 6·4 mmols. base are removed in the change from p_H 7·14 to 6·69. During this change 13 mmols. lactic acid have been formed, so that approximately half the base required for its neutralisation must have come from some other source than the phosphate, and therefore, on testing the base absorbing power of the warmed juice between 6·69 and 7·16, acid groups, other than phosphoric, which had been deprived of their base during the process of heating, secured the equivalent amount of base for their neutralisation and therefore raised the buffer capacity on this side.

Another example showing the changes which occur in juice on warming may be considered before the ultrafiltrates are dealt with.

Exp. 2. (Dog.)

Analyses. The nitrogen percentage of cold and warmed juice was 1·54.

	H_3PO_4 (inorganic) (molar)	Lactic acid (molar)
Cold	·025	·016
Warm	·049	·032

Reactions.

(*a*) Cold juice p_H 6·86

+ 6 mmols. acid, p_H 6·59

+12 mmols. base, p_H 6·94

Buffer capacity between 6·59 and 6·94 = 0·058*M* phosphate.

It would have been better to determine the capacity up to a more alkaline point as in the succeeding case.

(*b*) *Warm juice.*

Reaction p_H 6·36.

+ 24 mmols. base p_H 7·02.

Buffer capacity = 0·065*M* phosphate.

The buffer capacity of the cold juice between 6·59 and 6·94 is only slightly less than that of the warmed juice between 6·36 and 7·02, and in each case is much higher than the phosphate content.

If the cold juice be regarded as containing in the form of precipitable and hydrolysable phosphate the base present in a 0·049*M* concentration at p_H 6·97, then the ratio would be $\frac{\cdot 0248}{\cdot 0242}$, and at p_H 6·36 (warmed juice) $\frac{\cdot 0115}{\cdot 0375}$, or 13·3 mmols. base have passed from this system during heating, while 16 mmols. lactic acid were produced. The greater part of the base required for neutralisation could therefore be obtained from this source without encroaching on the colloidal or other base store, hence the amount of base which can be taken up between these two reaction points in the warmed juice should not differ much from that capable of being taken up by the cold juice.

Exp. 3. (Dog.) In this case juice and ultrafiltrates were both examined.

Analyses. The nitrogen percentage of juice was 1·36.

	H_3PO_4 (inorganic) (molar)	Lactic acid (molar)
Cold juice	·0214	·010
Ultrafiltrate	·0151	·015
Warmed juice	·0527	·035
Ultrafiltrate	·0341	·031

There is a marked discrepancy between the analyses of the juice specimens and their ultrafiltrates, especially affecting the phosphate values. Evidently the method employed for deproteinisation (trichloroacetic acid) removed more precipitable phosphate than passed through the celloidin plates. The juice specimens may, however, be compared with one another as may also the ultrafiltrates. The increments in phosphoric and lactic acids in the juice are approximately of the same order as in the ultrafiltrates.

Reactions (Fig. 3).

Cold juice p_H 6·20. As this reaction is much more acid than usual, in testing the buffer capacity, alkali was in the first place added until the p_H was 6·53.

(*a*) Cold juice p_H 6·53.

+ 12 mmols. base p_H 6·95.

Buffer capacity = 0·049*M* phosphate.

(*b*) Ultrafiltrate of cold juice—originally p_H 6·27, brought by addition of alkali to p_H 6·69. On the addition of 6 mmols. base the reaction shifted to p_H 7·40.

Buffer capacity = 0·016*M* phosphate.

(*c*) Warmed juice p_H 6·07. Alkali was added until the reaction was p_H 6·68. When 12 mmols. base were now added, the reaction shifted to 7·03 and, on a further addition of 12 mmols. to 7·41.

Buffer capacity = 0·064*M* phosphate.

(*d*) Ultrafiltrate of warmed juice p_H 6·18. Alkali was added until the reaction was 6·66. On addition to the solution at this reaction of 6 mmols. base, the reaction became 6·94 and on the addition of a further 6 mmols. rose to 7·30.

Buffer capacity = 0·034*M* phosphate.

The buffer capacities of the ultrafiltrate are evidently due to the inorganic phosphate present, while those of the juice specimens are much higher.

It is interesting to consider the possible source of the base necessary to neutralise the lactic acid produced in a cold juice at such an acid reaction as p_H 6·20.

1. At p_H 6·20, the cold juice, with 0·0214*M* phosphate, would have the ratio $\frac{\cdot0036}{\cdot0178}$ and therefore contain 25 mmols. base.

2. At p_H 6·07, in the warmed juice, with 0·0527*M* phosphate, the ratio would be $\frac{\cdot0071}{\cdot0456}$, and the content 59·8 mmols. base.

3. At p_H 6·20, the cold juice, with 0·0527*M* H_3PO_4 (precipitable + hydrolysable), would have the ratio $\frac{\cdot0090}{\cdot0437}$.

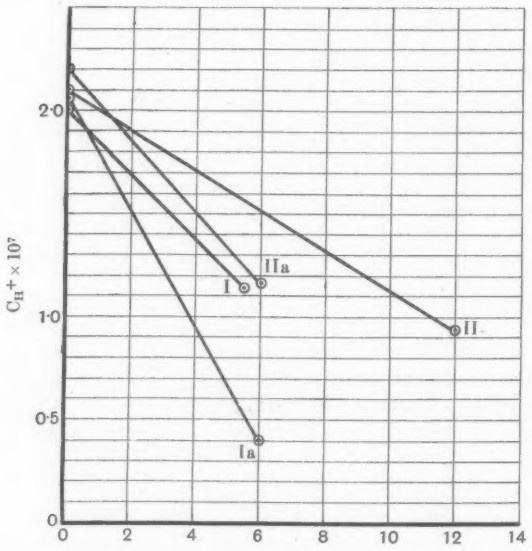

Fig. 3 (Exp. 3; Dog). I, Cold muscle juice; Ia, Ultrafiltrate of same; II, Heated juice; IIa, Ultrafiltrate of same.

Hence between p_H 6·2 and p_H 6·07 there would only be a removal of 1·9 mmols. base from the system 3.

During this very slight reaction change 25 mmols. lactic acid appeared and as only 1·9 base equivalents were obtainable from the phosphate system, and the 25 mmols. lactic acid must have secured at p_H 6·07 rather more than 24·5 mmols. base, the excess must have obtained its base from colloidal sources, as the ultrafiltrate of the heated juice shows no sign of a base-carrying system other than the inorganic phosphate. The heated juice ought therefore to have a corre-

spondingly higher buffer capacity than the cold. The rise in buffer capacity, as determined, corresponds approximately to an increase of 15 mmols. in the base-absorbing capacity of the warmed juice. The original higher value of the buffer capacity of the cold juice (over its phosphate content) is due to the preceding acid formation, which led to a reaction of p_H 6·20, having already removed base from base carrying systems other than the precipitable phosphate.

It is evident that the lactacidogen system does not contribute to the buffering value of the ultrafiltrate of the cold juice. Experiments are at present being carried out dealing with the condition of the forerunner of the precipitable phosphate in the juice.

Summary.

Horse muscle juice.

1. Reaction of cold juice at 18° varies from p_H 7·18–p_H 6·77, and of heated juice from p_H 6·63 to p_H 6·18. When the juice was expressed from muscle kept at room temperature p_H was 5·76.

2. On warming the juice there is always an increase in the inorganic phosphate which is more constant than the lactic acid increment.

3. The buffer values of the juice are higher than correspond to the actual phosphate concentrations, while the actual phosphate concentrations and buffer capacities of the ultrafiltrates correspond fairly closely.

Dog muscle juice.

1. Reaction of the cold juice varies from p_H 7·14–6·20 and of the heated juice from p_H 6·69–6·07.

2. On warming the juice there is always a greater increase in the inorganic phosphate and a more constant lactic acid increment than in the horse juice.

3. The buffer values of the juice are greater than the actual phosphate concentrations, except in one case where the lactic acid content of the cold juice was extremely low. The buffer values and the phosphate concentrations of the ultrafiltrates closely correspond to one another.

General conclusions.

1. In the expressed juice from frozen muscle there are present at least three systems which carry base in a readily disposable form, namely, the inorganic phosphates, the organic phosphate precursors and the colloidal or protein salts.

2. When lactic acid is produced as a result of heating the juice, the acid secures its base from all these systems, the buffering capacity depending especially upon the base derivable from the hydrolysed organic phosphate precursor and also from the constituents of the juice which do not pass into the ultrafiltrate.

3. If there is no inorganic phosphate increment accompanying the acid production, the buffering capacity is diminished, as the sole base-carrying systems which are then in evidence are the pre-formed inorganic phosphates and the proteins.

REFERENCES.

Embden, Kalberlah and Engel (1912). *Biochem. Z.* **45**, 45.
Embden, Griesbach and Schmitz (1914, 1). *Z. physiol. Chem.* **93**, 1.
Embden and Laquer (1914, 2). *Z. physiol. Chem.* **93**, 94.
—— —— (1921). *Z. physiol.* **113**, 1.
Fletcher and Hopkins (1906). *J. Physiol.* **35**, 247.
Harden (1923). Alcoholic Fermentation, 48.
Hill and Meyerhof (1923). *Ergebn. Physiol.* **22**, 229.
Meyerhof (1922). *Pflüger's Arch.* **195**, 22.
Michaelis and Davidsohn (1911). *Biochem. Z.* **33**, 456.
Parnas and Laska-Mintz (1921). *Biochem. Z.* **116**, 59.
Pauli (1920). Kolloidchemie der Eiweisskörper, 69.

CXXXIV. THE CATALYTIC ACTION OF TRACES OF IRON ON THE OXIDATION OF CYSTEINE AND GLUTATHIONE.

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The great acceleration produced in the atmospheric oxidation of cysteine by small quantities of iron was first shown by Mathews and Walker [1909], who observed that the reaction velocity was doubled by the addition of $M/100{,}000$ ferric chloride. These investigators also showed that the oxidation is inhibited by cyanides and nitriles, one molecule of hydrogen cyanide sufficing to inhibit the oxidation of one thousand molecules of cysteine. In its behaviour towards various reagents and in its susceptibility to changes in hydrogen ion concentration, the oxidation of cysteine closely resembles the oxidations taking place in the living cell, and has on this account received a considerable amount of attention.

Warburg and Sakuma [1923] put forward the theory that the so-called autoxidation of cysteine is in reality a catalysis by iron. In the oxidation of cysteine hydrogen cyanide inhibits the accelerating action of added iron, and its inhibiting effect on the oxidation of cysteine alone is ascribed to the same anticatalytic action on traces of iron present in the cysteine. It is assumed that the oxidation of cysteine takes place through the formation of an intermediate cysteine iron complex which is autoxidisable, but on addition of cyanide, a complex of cysteine, iron and cyanide is formed which is incapable of oxidation by molecular oxygen. By using quartz apparatus and taking special precautions to eliminate traces of iron, these workers claimed to have obtained samples of cysteine which were oxidised by atmospheric oxygen at an extremely slow rate.

Abderhalden and Wertheimer [1923, 1] attach great importance to the small residual oxidation which Warburg and Sakuma found to occur even in their purest samples of cysteine and attribute the inactivation by cyanide to another cause. The same investigators [1923, 2], working with glass apparatus, claimed to have prepared a specimen of cysteine free from iron, which was autoxidisable and the oxidation of which was still greatly inhibited by small quantities of cyanide. Consequently they assumed that the inactivation of the cysteine was not due to the action of the cyanide on iron. Mauthner [1912] found that cystine can be reduced by an aqueous solution of potassium cyanide, and Abderhalden and Wertheimer [1923, 2] confirmed this, finding that cystine is reduced by dilute potassium cyanide solution, the reduction

being detected by the development of the nitroprusside reaction. Dixon and Tunnicliffe [1923] found that the oxidation of cysteine is catalysed by cystine, probably by the formation of an intermediate cysteine-cystine complex. Abderhalden and Wertheimer ascribe the inactivation by cyanide to its reducing action on cystine, thus preventing the formation of the autocatalytic cysteine-cystine complex.

In view of the importance of information on the mechanism of the oxidation of sulphydryl compounds for a proper understanding of oxidations taking place in the living cell, it was thought desirable to carry out further work on the subject. In the first part of the present paper the effect of iron and cyanide on the oxidation of cysteine has again been studied and much of Warburg and Sakuma's work confirmed; while in the second part, the investigation has been extended to glutathione, the dipeptide which Hopkins has shown to play an important part in tissue oxidations.

Experimental.

I. CYSTEINE.

Preparation of iron-free cysteine.

The method used in the preparation of iron-free cysteine was essentially the same as that employed by Sakuma [1923]. The purification was carried out entirely in vessels of fused quartz, since the use of glass is liable to cause considerable contamination with iron, while difficulty was found in obtaining glazed porcelain quite free from iron. The quartz flasks were repeatedly boiled out with pure concentrated hydrochloric acid and were proved to be free from iron by the method employed by Sakuma. Water was distilled from a quartz distilling flask into a quartz receiver cooled with a stream of water, a platinum spiral being used to prevent bumping of the liquid. Pure concentrated hydrochloric acid was then boiled in the distilling flask, the side tube of the latter being placed just above the level of the water in the receiver. The distillation was continued until the acid in the receiver was six times normal. 20 cc. of this acid were evaporated to dryness in a quartz dish in presence of a crystal of pure potassium chlorate, the residue being dissolved in 1 cc. of water. 1 cc. of 10 % potassium thiocyanate solution was added together with 1 cc. of the 6*N* distilled acid, and the solution was viewed in a test-tube above a perfectly white background. By comparing this with a similar tube containing 3 cc. of pure water, any trace of yellow colour indicating the presence of iron could be observed. The final concentration of hydrochloric acid in the test-tube was 2*N*, at which concentration this test is most sensitive [Lachs and Friedenthal, 1911]. In a control experiment, the presence of 1/10,000 mg. of iron which had been added was readily detected. The distillation of hydrochloric acid was repeated until no trace of the iron reaction could be obtained. The water, ethyl alcohol and ammonia used in this work were freshly distilled in quartz vessels.

The starting product was *l*-cystine which was prepared from hair and twice recrystallised from 20 % hydrochloric acid. The cystine was reduced by heating for six hours on the water-bath with tin and 33 % hydrochloric acid in presence of a few drops of dilute platinum tetrachloride solution. During this reduction a slight evolution of hydrogen sulphide was observed. The tin was removed by diluting considerably with water and passing hydrogen sulphide. After filtering, the solution was evaporated to dryness *in vacuo*.

20 g. of the crude cysteine hydrochloride so obtained were dissolved in 100 cc. of water and saturated with pure hydrogen sulphide (prepared from antimony trisulphide and thoroughly washed with water). 2 mols. of finely powdered baryta were added, hydrogen sulphide passed for half an hour and the solution allowed to stand for some hours in a closed vessel. The dark green precipitate which separated was filtered off through an acid-extracted quantitative filter-paper and was found to contain large quantities of iron. Hydrogen sulphide was again passed through the filtrate and after standing, the solution was filtered into a quartz flask, further traces of the dark precipitate being so removed.

The solution was saturated with pure HCl gas, using the quartz distilling flask as a wash bottle, the side tube being placed just above the surface of the liquid and was then placed in a vacuum desiccator to remove hydrogen sulphide and evaporated to dryness on the water-bath in a quartz dish. The powdered residue was dried *in vacuo* over potassium hydroxide and extracted repeatedly with ethyl alcohol. The solution was evaporated to dryness *in vacuo* and the residue was recrystallised from alcohol. The yield of pure cysteine hydrochloride at this stage is small, but I have found that by the addition of a small quantity of chloroform (distilled in quartz vessels) to the mother-liquor a further quantity of cysteine hydrochloride is obtained which appears to be almost equally free from iron.

As found by Sakuma, the dried cysteine hydrochloride when titrated with iodine, required considerably more than the theoretical quantity. Doubtless this is due to the presence of a certain amount of free cysteine. A quantity of the cystine precipitated by iodine was dissolved in normal hydrochloric acid and the specific rotation measured in a 2 dm. tube. 0·0623 g. of cystine dissolved in 15·3 cc. gave a rotation of $-1{\cdot}825°$ which is equivalent to a specific rotation $[\alpha]_D^{20°} = -224°$. E. Fischer and Susuki gave $-222°$ as the specific rotation of *l*-cystine, while Sakuma obtained the value $-229°$.

Method of measurement of oxidation.

The rate of oxygen uptake by the reduced sulphydryl compounds was measured by means of the Barcroft micro-respirometer the bottles of which were made of fused quartz, sufficiently thick to withstand evacuation, and with necks ground to fit inside the glass stems, thus avoiding risk of impurities from the stopper grease.

Each bottle had a volume of 35 cc. and was calibrated to hold 3 cc. of

liquid. The apparatus was mechanically shaken in a water-bath maintained at constant temperature, and except where otherwise stated all measurements were made at 20° ± 0·1°.

In dealing with slow oxygen uptakes the apparatus was allowed to attain equilibrium by standing in the water-bath for about 7 minutes before taking readings. In measuring the more rapid reactions the method used by Dixon and Tunnicliffe [1923] was employed, the apparatus being evacuated, filled with pure nitrogen and allowed to attain equilibrium. It was then evacuated again and filled with air. The rate of oxygen uptake was found to be independent of the rate of shaking within wide limits.

The pure sulphydryl compounds were dissolved in water and brought to the required hydrogen ion concentrations by the addition of dilute ammonium hydroxide solution added from a quartz burette. The stopcock of the latter was large and well ground to eliminate the necessity for tap grease. The volume of alkali required was determined by a separate titration and was added to the solution in the Barcroft apparatus, together with sufficient water to make a total volume of 3 cc. The p_H was checked at the end of each experiment by means of Clark and Lubs' standard indicators.

The results obtained by direct measurement of the oxygen uptake agreed well with those obtained by shaking a larger volume of the solution in a quartz flask, pouring out small quantities at intervals and titrating with $N/100$ iodine solution from a micro-burette.

Rate of oxidation of purified cysteine.

Table I gives the results of a number of experiments with different samples of cysteine hydrochloride at various hydrogen ion concentrations. The rate of oxidation of iron-free cysteine is seen to be more than one hundred times slower than that of a crude sample. As is shown in Fig. 1 the velocity of oxygen uptake of pure cysteine is linear over a considerable period, and consequently can be expressed in the form $V = \frac{\text{(cmm. of oxygen)}}{\text{(mg. of cysteine, HCl) (minutes)}}$. The values so

Table I.

Preparation	mg. of cysteine, HCl in 3 cc. solution	p_H	Oxygen uptake in cmm. per hour	cmm./(mg. × mins.)
Cysteine (A)	10	7·7	6·22	0·0104
Cysteine (B)	10	7·7	2·49	0·0041
,,	12	7·7	4·21	0·0058
Cysteine (C)	9	7·3	3·32	0·0061
,,	10	7·7	4·60	0·0077
,,	10	7·7	2·80	0·0047
,,	10	7·8	5·00	0·0083
,,	13·5	8·0	4·80	0·0059
,,	36	8·0	17·5	0·0081
,,	10	7·6	2·32	0·0039
Crude cysteine	10	8·0	380·0	0·63
Cysteine (C) + $M/1000$ HCN	10	7·6	1·86	0·0031

obtained are given in the last column of Table I and are of the same order as the average values obtained for pure cysteine by Warburg and Sakuma.

The upper curve in Fig. 1 shows the oxygen uptake of a sample of pure cysteine hydrochloride, while the lower curve shows the effect of the addition of $M/1000$ hydrogen cyanide. Potassium cyanide solution together with the equivalent amount of hydrochloric acid was added to the acid cysteine solution before adjusting the p_H with ammonia. Measurements were taken over a period of 24 hours and it will be observed that the rate of oxidation of pure cysteine is linear over a considerable part of the curve, showing a slight falling off after 9 hours, while the rate of uptake of cysteine + cyanide is linear throughout the whole 24 hours. This will be referred to later.

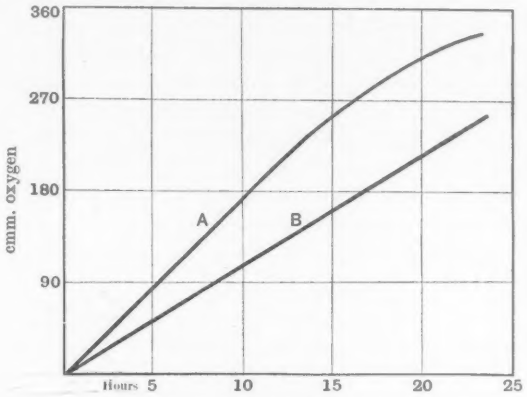

Fig. 1. Absorption of oxygen by pure cysteine.
A. 36 mg. pure cysteine hydrochloride.
B. 36 mg. „ „ „ + $M/1000$ HCN.
$p_H = 8{\cdot}0$. Temp. = 20·0°.

Catalytic action of traces of iron on the oxidation of purified cysteine.

The oxidation of pure cysteine is very strongly catalysed by iron, and Warburg and Sakuma found that a marked increase in velocity is produced even by the addition of a few 1/100,000ths of a mg. of iron to the solution. These workers expressed the catalytic activity of the iron in the form,

(increase of oxygen uptake in cmm.) ÷ (mg. of added iron)
× (time in minutes) = n_{Fe},

and found the average value of this quotient to be 1700.

In my earlier experiments, using ferric chloride, the values obtained were considerably lower than this and were very variable. It was thought that this might be due to the inactivation of the iron on standing in very dilute solution, possibly owing to some physical change in the ferric hydroxide formed by hydrolysis; and on using a freshly prepared solution of ferric chloride and diluting immediately before each experiment, consistent results were obtained.

Ferrous iron, added as ferrous ammonium sulphate, was found to be equally active.

Table II.

Exp.	mg. of cysteine, HCl	p_H	Initial O_2 uptake in cmm. per hr.	mg. of iron added	Final O_2 uptake in cmm. per hr.	n_{Fe}
1	10	7·7	6·6	0·0001 (ferric)	15·6	1500
2	12	7·7	4·2	0·0002 „	19·8	1300
3	10	7·7	2·8	0·0001 (ferrous)	12·8	1700
4	13·5	8·0	4·8	0·0004 (haematin)	23·2	770
5	10	8·0	7·2	0·0008 „	47·7	840

The first part of Table II shows the catalytic effect of ferric and ferrous ions on cysteine oxidation and the values obtained for n_{Fe} agree well with those found by Warburg and Sakuma.

In the second part of the same table is shown the catalytic effect of the iron in haematin. The haematin was prepared by washing crystals of pure haemin (prepared from ox blood) successively with alcohol, pure concentrated HCl, and distilled water, to remove any traces of free iron, dissolving in two equivalents of $N/2$ ammonium hydroxide and diluting with water. The iron in haematin, while less active as a catalyst than inorganic iron, appears to be less sensitive to the action of cyanide.

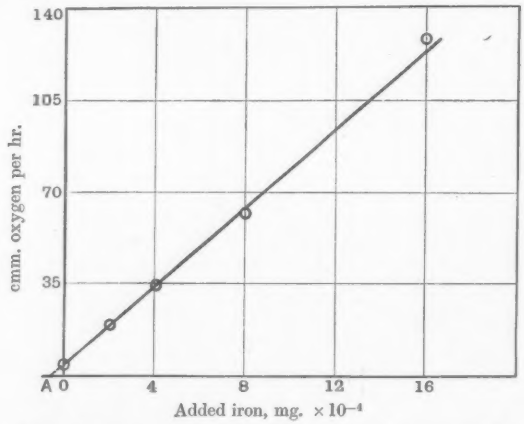

Fig. 2. Effect of increasing concentration of iron on the rate of oxygen uptake by pure cysteine 12 mg. pure cysteine hydrochloride. Iron added as $FeCl_3$.
$p_H = 8·0$. Temp. = 20·0°.

Fig. 2 shows the effect of adding increasing amounts of iron (as ferric chloride) on the velocity of oxygen uptake of pure cysteine. The increase in velocity is seen to be directly proportional to the quantity of iron added. The initial velocity of uptake of the pure cysteine was 4·2 cmm. per hour and if this residual uptake were assumed to be due to a trace of iron remaining in the purified cysteine the amount of this remaining iron would be given by the

distance AO, the curve being produced to meet the abscissa at A. The distance AO would then represent 0·00005 mg. of iron present in 12 mg. of cysteine hydrochloride.

In order to test this assumption, 0·10 g. of the same sample of cysteine hydrochloride was ashed in a quartz crucible which was then washed out with 1 cc. of hot 6N hydrochloric acid. This was added to 2 cc. of 5 % KCNS solution and comparisons made with solutions containing known amounts of iron. In this way the amount of iron was found to be about 0·0001 mg. (certainly less than 0·0002 mg.). This represents only 0·00001 mg. in 12 mg. of cysteine hydrochloride, and consequently, the residual uptake does not appear to be entirely due to the presence of iron.

This is confirmed by the last experiment in Table I which shows that the inhibiting action of M/1000 HCN on the oxidation of pure cysteine is small, the value of V being reduced from 0·0039 to 0·0031. If the oxygen uptake of pure cysteine were entirely due to traces of residual iron, it would be expected that M/1000 HCN would produce a considerably greater inhibition, assuming Warburg's theory that iron is inactivated by cyanide. Evidence in favour of the correctness of this assumption will be given later in the paper.

Hence it would appear either that traces of catalysts other than iron are present in the purified cysteine or that to a minute extent cysteine is strictly autoxidisable.

II. GLUTATHIONE.

Preparation of iron-free glutathione.

The purification of glutathione involved the use of pure mercuric sulphate reagent and pure barium hydrosulphide solution.

The mercuric sulphate was prepared by heating together 18 g. of mercury with 27 g. of sulphuric acid in a quartz dish. Both the mercury and the sulphuric acid were previously distilled from quartz. 15 g. of the crystalline products were added to 150 cc. of water and pure sulphuric acid was added until the mercuric sulphate was just dissolved.

The solution then contained about 9 % of sulphuric acid.

The barium hydrosulphide was prepared by shaking excess of finely powdered baryta with water and saturating with pure hydrogen sulphide. The solution was poured off from the excess of baryta, allowed to stand overnight and filtered to remove traces of iron.

2 g. of oxidised glutathione prepared from yeast were dissolved in 10 cc. of water. The solution was saturated with pure hydrogen sulphide, a total of 7 g. of finely powdered baryta being meanwhile added at intervals. The solution was allowed to stand overnight in a closed vessel, and a considerable quantity of a dark greenish precipitate was filtered off and washed with a little barium hydrosulphide solution. 3 g. of finely powdered baryta were added to the filtrate and washings and hydrogen sulphide passed for ½ hour.

After standing in a closed vessel for 2 days the solution was filtered into a quartz flask, diluted with 100 cc. of water, made just acid with sulphuric acid and again filtered. A slight excess of mercuric sulphate reagent was added and the precipitate filtered off, washed with water, suspended in 75 cc. of water and decomposed with hydrogen sulphide. The precipitated mercuric sulphide was allowed to settle and the suphuric acid was removed exactly with barium hydrosulphide.

The solution which was then still distinctly acid to litmus, was filtered into the quartz distilling flask and evaporated down to about 5 cc. in hydrogen at 40 mm. pressure.

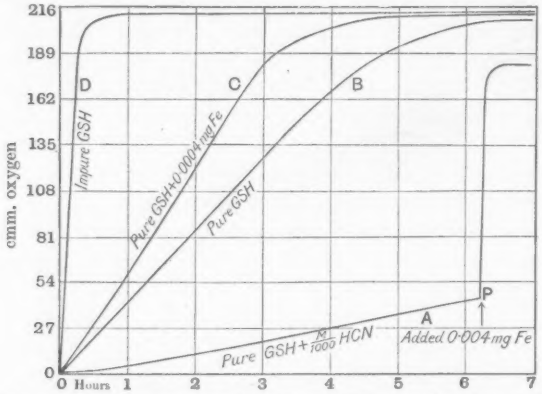

Fig. 3. Comparison of rates of oxygen uptake of pure and impure glutathione. Catalytic effect of a trace of iron on the rate of oxygen uptake of pure glutathione, and the inhibiting effect of hydrogen cyanide.

A. 16 mg. pure glutathione + $M/1000$ HCN.
B. 16 mg. „ „
C. 16 mg. „ „ +0·0004 mg. Fe (as $FeCl_3$).
D. 16 mg. crude „
$p_H = 8{\cdot}0$. Temp. = 20·0°.

The product was poured into 80 cc. of absolute alcohol and allowed to stand for 24 hours. The alcohol was then poured off and 80 cc. of fresh alcohol added. The glutathione was allowed to stand under alcohol until it became quite friable. The alcohol was then poured off and the pure gluthathione dried in a vacuum desiccator.

By titration with iodine the product was found to contain 66 % of the reduced form of glutathione.

The dark green precipitate which formed on passing hydrogen sulphide through the solution of crude glutathione in baryta was found after washing to contain large quantities of iron, the only other metal found being barium.

The precipitate was dissolved in acid and the iron estimated; 0·0178 g. of Fe_2O_3 were obtained from the 2 g. of glutathione. This is equivalent to over 0·6 % of Fe in the crude glutathione.

Rate of oxidation of glutathione.

The rate of oxidation of purified glutathione is shown by curve *B* in Fig. 3. It will be observed that as in the case of iron-free cysteine the velocity of oxygen uptake is linear over a considerable part of the curve. By comparison with curve *C* it is evident that the oxidation of purified glutathione is strongly catalysed by minute quantities of iron, the rate of uptake of oxygen being increased from 42 cmm. to 57 cmm. in the first hour, by 0·0004 mg. of iron.

Curve *D* shows the rate of oxidation of an impure specimen of glutathione under the same conditions. The oxygen uptake is extremely rapid, the glutathione being almost completely oxidised in half an hour. Comparing this with curve *B*, it is seen that by removing iron from glutathione its initial velocity of oxygen uptake is reduced from about 760 cmm. to 42 cmm. per hour.

Curve *A* shows the effect of the addition of *M*/1000 HCN to the purified glutathione. The rate of uptake is still further reduced, though it is considerably greater than that of pure cysteine in presence of *M*/1000 cyanide.

At point *P* on the curve 0·004 mg. of iron was added which produced an enormous acceleration in the rate of oxygen uptake, the oxidation being complete in a few minutes. The total uptake in this case is somewhat less than theoretical, owing partly to a certain amount of oxidation having taken place on the addition of the iron before the apparatus could be evacuated and brought to equilibrium in nitrogen, and partly to the removal of 0·1 cc. of solution before adding an equal volume of the iron solution, in order to keep the volume of liquid in the apparatus constant.

As was found in the case of pure cysteine + *M*/1000 cyanide, the rate of oxygen uptake of pure glutathione + *M*/1000 cyanide is perfectly linear.

The effect of varying concentrations of cyanide on impure glutathione was then tried. Fig. 4 shows that increasing inhibition of the rate of oxidation is brought about by increasing cyanide concentration. Here again the velocity of uptake is seen to be practically linear, and to remain so until the oxidation is almost complete. The significance of these linear curves will be discussed later.

In a similar experiment using a sample of impure glutathione which was known to contain less iron than that used in the experiments shown in Fig. 4, the actual reduction in the rate of oxidation brought about by varying concentrations of cyanide was relatively greater.

Warburg and Sakuma showed that from p_H 9·2 to 10·2 the oxidation of cysteine was inhibited by *M*/10 sodium pyrophosphate to the same extent as by *M*/1000 hydrogen cyanide. They assume that this is due to the formation of a non-catalytic complex of iron with the pyrophosphate, this complex being more stable at an alkaline p_H. I find that the oxidation of glutathione is also inhibited by pyrophosphate and Fig. 5 shows that a considerable inhibition is produced even by *M*/50 pyrophosphate at p_H 7·6, and that the rate of oxygen uptake becomes linear over a considerable period.

It was thought to be of interest to try the effect of the addition of cyanide to the system oxidised glutathione + thermostable tissue described by

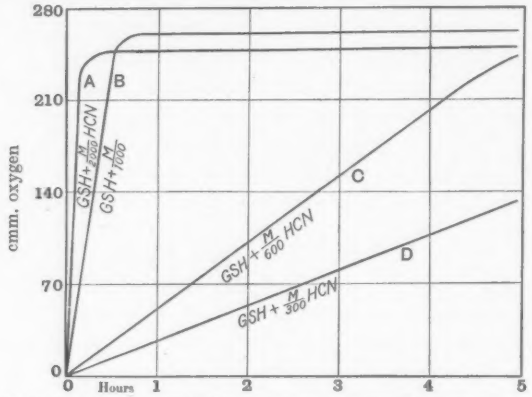

Fig. 4. Inhibition of the rate of oxidation of unpurified glutathione by hydrogen cyanide.
A. 16 mg. unpurified glutathione + $M/2000$ HCN.
B. 16 mg. ,, ,, + $M/1000$ HCN.
C. 16 mg. ,, ,, + $M/600$ HCN.
D. 16 mg. ,, ,, + $M/300$ HCN.
$p_H = 7{\cdot}6$. Temp. = 20·0°.

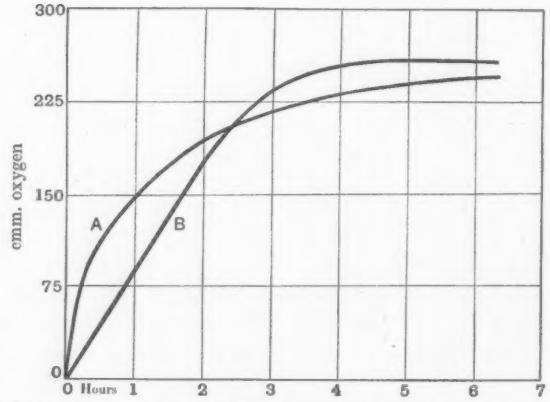

Fig. 5. Inhibition of the rate of oxidation of unpurified glutathione by pyrophosphate.
A. 15 mg. unpurified glutathione in phosphate buffer.
B. 15 mg. ,, ,, + $M/50$ sodium pyrophosphate in phosphate buffer.
$p_H = 7{\cdot}6$. Temp. = 20·0°.

Hopkins and Dixon [1922]. The tissue was prepared by boiling finely chopped rat muscle with water, treating with alcohol and drying *in vacuo*. The finely powdered product when shaken in buffer solution at p_H 7·6 shows only a small uptake of oxygen. The tissue is capable of reducing oxidised glutathione,

and on adding glutathione in the oxidised form, the system takes up oxygen rapidly to the extent of about 400 to 500 cmm. per gram of dry tissue. Fig. 6 shows the effect of hydrogen cyanide on this uptake of oxygen. A definite inhibition is evident, though this is much less than that produced on the rate of oxidation of reduced glutathione alone, and it will be seen that in the presence of tissue, the curves obtained with cyanide are no longer linear.

It will be noticed that in presence of cyanide the total uptake of oxygen by the system is somewhat increased. This was found to be the case in a number of experiments. Abderhalden and Wertheimer [1923, 2] have shown that cystine oxidises dilute potassium cyanide solution and it is possible that

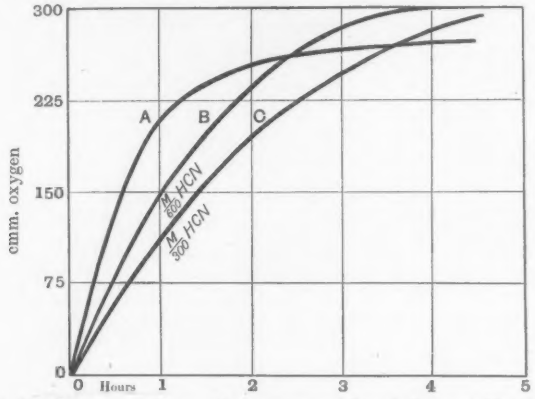

Fig. 6. Effect of hydrogen cyanide on the oxygen uptake of thermostable tissue + oxidised glutathione.

A. 0·5 g. tissue + 16 mg. oxidised glutathione (neutralised).
B. 0·5 g. „ + 16 mg. „ „ + M/600 HCN.
C. 0·5 g. „ + 16 mg. „ „ + M/300 HCN.
In phosphate buffer at $p_H = 7·6$. Temp. = 34·0°.

the increased oxygen uptake may be due to a similar reaction between the cyanide and oxidised glutathione, leading to the formation of more reduced glutathione and consequently to a higher oxygen uptake. It may be mentioned that Ellinger and Landsberger [1922] claim that hydrogen cyanide may be oxidised on the muscle surface.

Discussion.

It would appear from the foregoing experiments that in their behaviour during oxidation cysteine and reduced glutathione are essentially analogous. The normal rate of oxidation of both compounds is greatly reduced by careful purification, and both show a marked acceleration in presence of iron and an inhibition in presence of cyanide. On adding iron to a solution of reduced glutathione a deep violet colour is produced similar to that given with iron by cysteine and if the solution be shaken in air, the colour persists until the

glutathione is completely oxidised. It appears probable that, as in the case of cysteine the acceleration in the rate of oxidation of glutathione produced by iron is due to the formation of an autoxidisable complex of glutathione with iron.

The difference between the aerobic oxidation of cysteine and that of glutathione appears to be one of degree rather than kind, glutathione being oxidised more rapidly than cysteine. That this is not a fundamental difference is shown by Dixon and Tunnicliffe who have found that in the anaerobic oxidation of these compounds, using methylene blue instead of oxygen as the hydrogen acceptor, the order is reversed, cysteine being oxidised more rapidly than glutathione.

There appears to be much evidence in favour of the assumption that the inhibiting action of cyanide on oxidations is due to its combining with traces of catalytic iron to form a non-catalytic complex. Warburg and Brefeld [1924] have shown definitely that in the catalytic oxidation of a solution of leucine by iron on the surface of charcoal, the inhibition caused by hydrogen cyanide is due to its inactivating action on the iron, and it seems highly probable that the same is true in the case of the oxidation of cysteine and glutathione. Further evidence for this point of view is afforded by the observation mentioned earlier in the paper that with samples of cysteine and glutathione containing small amounts of iron the relative inhibition by cyanide was less than with those containing more iron.

Meyerhof [1923] has shown that the inhibition by cyanide of the catalytic action of copper on the oxidation of thioglycollic acid is due to the formation of a CuCN complex, and the action of cyanide on iron is believed to be analogous to this.

Definite evidence for the theory of cyanide inactivation of iron is afforded by Warburg's observation that sodium pyrophosphate which, like cyanide, is known to form a complex with iron, also inhibits the oxidation of cysteine.

In Fig. 5 it was shown that sodium pyrophosphate inhibits glutathione in a similar manner, and further that, as in the case of cyanide inhibition, the curve becomes linear.

It will be observed that in oxidations taking place in presence of a considerable amount of ionised iron, the form of curve for oxygen uptake is such as would be expected if the concentration of sulphydryl compound formed the limiting factor in the reaction velocity. The falling off in velocity with decreasing concentration of sulphydryl compound is of course modified by the autocatalytic nature of the reaction.

On the other hand, in those oxidations in which the quantity of ionised iron in solution is very small, owing either to careful purification or to complex formation with cyanide or pyrophosphate, the curves are linear until oxidation is almost complete. The reason for these two different types of curve seems to be as follows.

The sulphydryl compounds are apparently incapable of combining directly

with molecular oxygen, the latter requiring first to be activated by means of iron. It has not been definitely proved, however, that the very slow rate of oxidation of the purified compounds is not due to their autoxidation, although it may well be due to traces of catalysts which have escaped removal.

Suppose that cyanide forms a complex either with the catalytic iron alone, or more probably with iron together with the sulphydryl compound. Let us assume that this complex is slightly dissociated and that a very small concentration of ionised iron is thus present in solution. It is evident that with such a small concentration of iron, the relatively large quantity of sulphydryl compound would be capable of taking up the activated oxygen more quickly than it could be formed. Consequently the rate of activation of oxygen by iron becomes the limiting factor in the reaction, and this rate being constant, the oxygen uptake curve is linear. When the oxidation is almost complete, the concentration of sulphydryl compound becomes so small that the rate at which it can take up active oxygen becomes the limiting factor, and consequently a progressive falling off in velocity then takes place.

This theory explains the curves in Fig. 4 which show that increasing the concentration of cyanide produces a progressive increase in the inhibition. If the cyanide complex with iron were entirely undissociated, it would be expected that on increasing the concentration of cyanide, the amount of inhibition would reach a maximum when all the free iron was taken up.

On the other hand, if we assume that the complex is slightly dissociated, it is evident that increasing the concentration of cyanide will decrease the amount of this dissociation and so bring about an increased inhibition of oxidation.

In the same way if the concentration of iron in solution be rendered very small by careful purification of the sulphydryl compound, the rate of activation of oxygen is again the limiting factor and again linear curves are obtained.

On the other hand, when a comparatively large amount of iron is free in solution as in the case of solutions of ordinary samples of cysteine and glutathione, excess of active oxygen is available throughout the whole reaction and the limiting factor becomes the rate of uptake of active oxygen by the sulphydryl compound. Therefore as the concentration of sulphydryl compound falls off, a progressive decrease in velocity takes place, though this decrease is modified by the catalytic effect of the oxidised form as shown by Dixon and Tunnicliffe.

In the case of the system glutathione + tissue, the inhibiting effect of hydrogen cyanide on the rate of oxygen uptake is much less marked than in the case of the oxidation of reduced glutathione in the absence of tissue. It was recorded earlier in the paper that the catalytic effect of iron in haematin is inhibited to a smaller extent by cyanide than that of inorganic iron, and as it is probable that oxidations in the tissue may be catalysed by organic iron compounds allied to haematin, the smaller inhibition by cyanide would be expected.

It is not difficult to understand why the iron in the haematin compound should be active as a catalyst while the cyanide compound with iron is inactive. In cyanide-iron compounds, in presence of oxygen, the ferrocyanide form is known to be more stable than the ferricyanide form, and hence the ferrocyanide compound, when once formed, would show no tendency to take up oxygen. In haematin, however, the ferric compound is the more stable in presence of oxygen, and consequently as soon as this is reduced to the ferrous compound by the sulphydryl group, it becomes oxidised again by the air, the iron in haematin thus acting as an oxygen carrier.

Further work is being carried out with a view to finding whether there is any connection between the catalysis of the oxidation of sulphydryl compounds by iron and by disulphides.

Summary.

1. The purification of cysteine and glutathione has been carried out using quartz vessels and taking special precautions to remove iron. Much of the work of Warburg and Sakuma on cysteine has been confirmed.

2. The rates of atmospheric oxidation of both cysteine and glutathione are found to be very greatly reduced by the removal of traces of iron impurities.

3. It has been shown that the addition of quantities of iron of the order of 1/10,000 mg. produces a marked increase on the rate of oxidation of the purified cysteine and glutathione.

4. Evidence has been given that the inhibition in the oxidation of cysteine and glutathione by hydrogen cyanide is due to its forming a complex with the catalytic iron.

5. The velocities of oxygen uptake in the case of the purified compounds alone, and in the case of the unpurified compounds in the presence of cyanide, have been found to be linear.

6. It has been found that the iron in haematin is also capable of catalysing these oxidations.

I wish to express my sincere thanks to Professor F. G. Hopkins for his interest and encouragement throughout this work.

I also wish to thank Mr R. Hill for providing me with a sample of pure haemin.

References.

Abderhalden and Wertheimer (1923, 1). *Pflüger's Arch.* **200**, 650.
—— —— (1923, 2). *Pflüger's Arch.* **198**, 126.
Dixon and Tunnicliffe (1923). *Proc. Roy. Soc. London*, B, **94**, 266.
Ellinger and Landsberger (1922). *Z. physiol. Chem.* **123**, 246.
Hopkins and Dixon (1922). *J. Biol. Chem.* **54**, 527.
Lachs and Friedenthal (1911). *Biochem. Z.* **32**, 130.
Mathews and Walker (1909). *J. Biol. Chem.* **6**, 299.
Mauthner (1912). *Z. physiol. Chem.* **78**, 32.
Meyerhof (1923). *Pflüger's Arch.* **200**, 1.
Sakuma (1923). *Biochem. Z.* **142**, 68.
Warburg and Brefeld (1924). *Biochem. Z.* **145**, 461.
Warburg and Sakuma (1923). *Pflüger's Arch.* **200**, 203.

CXXXV. THE EFFICIENCY OF VARIOUS SUGARS AND THEIR DERIVATIVES IN RELIEVING THE SYMPTOMS CAUSED BY INSULIN IN MICE.

By PERCY THEODORE HERRING,
JAMES COLQUHOUN IRVINE and JOHN J. RICKARD MACLEOD.

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PART I.

EXPERIMENTAL OBSERVATIONS.

THE object of the experiments recorded in this paper was primarily to ascertain the effects of alterations in the molecular constitution of glucose upon the efficiency of the parent sugar in alleviating the symptoms caused by insulin. The work was initiated at the suggestion, and under the direction, of Professor Macleod, and was rendered possible by the collaboration of Principal Irvine, who supplied the necessary chemical compounds. Some of these were available in small quantities only, and as the tests had to be made upon a sufficient number of animals, mice were chosen for the purpose. The results obtained rendered it advisable to extend the observations to the effects of other carbohydrates, some of which have already been investigated by Noble and Macleod [1923] in rabbits.

The mice were healthy young adults, varying in weight from 16 to 35 g., and were of both sexes. The animals were well fed, and no preliminary starvation or other procedure for diminishing their glycogen content was adopted.

The insulin employed was that of Eli Lilly and Company, and contained 20 units per cc. The amount injected hypodermically was intended to cause convulsions without being fatal, but experience shows that the attainment

[1] The experimental part of the work was carried out in the Physiology Department of the University of St Andrews, being part of the work done by P. T. Herring for the Medical Research Council.

of such a standard is impossible, great variations occurring in different animals as the result of the same dosage. The standard solution of insulin was diluted to one in twenty-five with 0·05 % HCl, and the dosage of the latter varied between 3 and 5 minims.

The mice were selected in batches of three of as nearly the same weight as possible, but the three were usually of different colours to facilitate recognition when in convulsions. Each mouse of the batch received a hypodermic injection of the same amount of insulin. The animals were then placed in open boxes in a large incubator which was kept at a temperature between 20° and 30°. The observations were as far as possible conducted in a warm atmosphere throughout.

The results of the injection were very variable in the mice of the same batch. Not infrequently one or more of the animals showed no symptoms even when they were severe in another. Occasionally all were affected alike. The most severe symptoms might occur in the heaviest animal. The colours of the mice employed were white, black and white, black, fawn, fawn and white, and brown. No one colour appeared to be associated with a greater or a less resistance to the effects of the insulin.

When symptoms had definitely developed the animal was carefully watched for a time and then a hypodermic injection of the test substance was given. This was frequently repeated after an interval and the results noted.

The symptoms produced in mice by an injection of insulin vary somewhat in detail, but are, on the whole, very much alike. Their onset usually occurs about three-quarters to one hour and a half after the injection, but may be delayed for two hours or longer. The animal becomes irritable and restless. The tail stiffens and may be held arching over the back or pointing upwards and backwards. A slight tap on the box or sudden noise makes the mouse jump. This stage is quickly followed by convulsions, of which there are two types. In the more common form the mouse springs into the air and runs about wildly, or rolls over and over sideways, the muscular contractions being of a clonic character. In the other, and not infrequent form, the mouse has a tonic spasm affecting all its muscles. The back is arched, the limbs extended, and head retracted. The spasm lasts for a few seconds and the animal falls over on its side. In the intervals between the convulsions the animal is limp and lies with legs extended in the prone position. Weakness of the postural muscles is very prominent, and the sprawling thus occasioned is a prominent phenomenon after the first stage of irritability is over. Occasionally the sprawling precedes the convulsions. At this stage handling the animal usually sets up convulsions. Voluntary movements gradually become weaker, but the mouse, if placed on its back, regains the prone position and may drag itself about with its hind limbs extended and abducted. The convulsive stage may be prolonged or may be very short. If the amount of insulin injected has been sufficiently large the mouse gradually loses all power of movement and passes into the stage of collapse and coma. The animal now lies in whatever position

it is placed, and makes no attempt to right itself. The body is limp, the eyes protrude, and the pupils are widely dilated. Respirations become slower and of a convulsive inspiratory type. Death soon follows unless the mouse is resuscitated by the hypodermic injection of an active sugar.

Occasionally convulsions do not occur or are so slight that they are not noticed, and the animal passes rapidly into the comatose state.

If the dose of insulin administered has not been too large spontaneous recovery from the convulsive stage is usual but takes place slowly with frequent relapses. This fact has to be borne in mind in drawing conclusions as to the efficiency of substances tested for their capability of relieving symptoms and effecting a permanent cure. It is known that the hypodermic injection of glucose relieves the symptoms of insulin very promptly, and is effective even in the late stages of insulin coma. Moreover glucose effects a permanent cure if given in sufficient amount. Noble and Macleod have pointed out that certain substances, e.g. fructose and galactose, are capable of alleviating the symptoms of insulin in rabbits, but are incapable of effecting a complete recovery, the animal showing relapses after temporary improvement. In claiming for a substance an efficiency in relieving the condition produced by insulin which is comparable with that of glucose it is therefore necessary to show that it can produce not only temporary relief but can also effect a permanent recovery. For this purpose a more than lethal dose of insulin is required, and the substance tested must be capable of rapidly producing a complete cure.

In the following experiments the action of substances tested has been recorded on mice which were subject to insulin symptoms of various degrees of severity. Where any doubt existed as to the efficiency of the substance tested the experiments have been more numerous. Where the results were decidedly negative a few experiments have been deemed sufficient.

The substances tested were made up in distilled water and were of such concentration as to be as nearly equivalent as possible to that of the standard, i.e. glucose, 15 %. In some cases the solubility of the substance did not admit of this concentration, but in these and other variations below the standard the dose administered by hypodermic injection was correspondingly increased.

Substances investigated.

I. *Unsubstituted Reducing Sugars.*

1. *Glucose.* Three minims of a 15 % solution of glucose was usually effective in resuscitating a mouse even from deep insulin coma. The injection of glucose failed only in a few cases, and in all of these the animals were moribund at the time of the injection, death occurring before the glucose had time to be absorbed. Recovery to glucose was usually very rapid, a few minutes sufficing to restore the animal from deep coma to an apparently normal condition. Relapse may occur even after glucose, and if the dose of insulin has been a

large one the tendency to relapse lasts for some hours, but the condition is at once relieved by a further injection.

In recovery from insulin coma one of the first changes noticeable is the reduced protuberance of the eyeballs and the occurrence of winking movements by the eyelids. This is sometimes followed by convulsive movements which may be similar to those of the pre-coma stage but are of short duration only. Such convulsions do not always take place in recovery. The convulsive movements in recovery are more often those of a spasmodic contraction of the flexor muscles and differ from the earlier convulsions in which contraction of the extensors predominates. The fore limbs are tightly flexed against the chest, the toes strongly flexed, and the body curled up. The mouse soon struggles into a prone position, the irritability disappears, as does also the weakness of the postural muscles. In a very short time the animal resumes a normal posture, and, except for some lethargy from which it is easily aroused, appears to be fully restored.

2. *Mannose.* A 15 % solution of this sugar was used in place of glucose to resuscitate animals in which other substances had failed. Mannose was used on nine occasions. In two of these the mice were moribund in the comatose state with convulsive inspirations, a condition which is rapidly fatal if untreated. 5 minims of the mannose solution in each case brought about a complete recovery in 3 minutes which was permanent. In three mice the irritability and sprawling which had not been relieved by fructose were at once permanently abolished by 4 minims of mannose. In the remaining four mice the convulsive stage was at once abolished and permanent cure effected by similar doses of mannose after failure by galactose in three cases and glucosan in one.

The mannose was uniformly successful and appeared to be no less efficient than glucose.

3. *Fructose.* This sugar has been used in a 15 % solution in 20 mice. In nine of these its action was tested after other substances had failed to produce any effect upon the insulin symptoms. In the other 11 mice fructose was the first substance employed. The sugar was tested upon mice showing insulin symptoms of various degrees of severity. The results show that fructose is not always successful in relieving the symptoms, but that it does so in a majority of instances, though not so quickly as glucose or mannose. Larger doses are necessary and the beneficial effects are not so complete and permanent. In three mice fructose had no apparent effect but recovery was immediate and permanent to a small dose of glucose.

In one of these, black mouse, weight 29 g., a 4 minim dose of fructose failed to induce any sign of recovery from the comatose state, but 6 minutes later complete recovery was rapidly produced by the injection of 2 minims glucose. Another mouse of the same batch, white, 27 g., received the same dosage of insulin, and passed through the convulsive stage to that of coma. A dose of 5 minims fructose failed to produce any improvement, and the

mouse was resuscitated three minutes later by the injection of 2 minims glucose solution. Both mice recovered very rapidly to glucose and the recovery was permanent in each case. Fructose in a dose which was double or more that of glucose failed to induce any recovery in 6 minutes in the first mouse and 3 minutes in the second.

Definite improvement to fructose took place in 17 mice, but in 10 of them an injection of glucose was eventually necessary to make the recovery complete. The remaining seven recovered without further treatment, but as several mice receiving the same dose of insulin and showing the same symptoms also recovered without even fructose it cannot be claimed that the seven mice owed their recovery to the fructose. The temporary alleviation often given by an injection of fructose may, however, be the determining factor in recovery when the dose of insulin has not been too great.

The experiments show that fructose may have a temporary alleviating action, but fails in itself to produce a complete recovery. The results obtained in mice with fructose are similar to those found in rabbits by Noble and Macleod.

4. *Galactose.* A 15 % solution of this sugar was employed in 18 mice. No improvement was noted in eight, and in three of these death occurred after doses of galactose amounting to 13 minims in one, 10 minims in another, and 5 minims in the third. The other five were resuscitated, two by glucose 4 and 5 minims respectively, and the remaining three by 5 minims maltose each. Nine mice showed definite improvement after the injection of galactose, but the improvement was of short duration. Improvement was often manifested to repeated doses, but relapse always followed, and the animals were eventually resuscitated by small doses of glucose, mannose or maltose. Only one mouse showed a gradual and permanent recovery after a dose of 5 minims galactose, and it would probably have recovered without treatment.

The temporary cessation of convulsions after an injection of galactose was a striking phenomenon in many cases, but relapse occurred within a few minutes. From 10 to 16 minims of galactose were given in some instances in two or more doses, and while producing a temporary short alleviation even these amounts were incapable of effecting a complete recovery. In two mice, after the failure of galactose, two doses each of 5 minims fructose were successful in effecting a complete recovery. These mice had not had large doses of insulin, and fructose proved to be more efficient than galactose in alleviating the symptoms.

Galactose is not a sugar which is capable of effecting a complete recovery from insulin symptoms in mice. It is not always successful in alleviating the symptoms even, and when it does so its action is evanescent. The temporary beneficial action of galactose is best shown in mice which are in the convulsive stage and have not received a large dose of insulin. It fails to resuscitate mice which have passed into the comatose state, and is less effective than fructose. Noble and Macleod found that galactose, like fructose, had a temporary

alleviating action in rabbits. The results of these experiments on mice show that galactose acts in a way like fructose but is much less efficient. Galactose cannot take the place of glucose or mannose.

5. *Maltose.* The maltose employed was of exceptional purity and made up in a 15 % solution. 16 mice were injected, the dosage being 5 minims in each case, the same amount being repeated in three only. Six of the mice received maltose alone, the other ten were treated with maltose after other substances had failed to give any relief from the symptoms. The maltose effected a complete and permanent cure in 15 of the mice, but failed in one in which death occurred 3 minutes after the injection. This mouse was moribund at the time of injection and had previously received two doses of 5 minims each of sucrose. An injection of glucose or mannose might have been effective. There is some indication that the action of maltose is somewhat slower than that of glucose or mannose.

The conclusion is reached that maltose is an effective sugar and can rank with glucose and mannose though its efficiency is less. Larger doses have to be employed and there is often some delay in its action. Noble and Macleod found maltose to be somewhat similar in its action to fructose and galactose in rabbits, and that it could not take the place of glucose. The experiments now done on mice appear to show that in these animals maltose is not much less efficient than glucose.

6. *Lactose.* A 15 % solution of this sugar was used in four mice only. Three of them received 10 minims in two doses of 5 minims each, the fourth had three doses of 5 minims each. In no case was there the slightest effect upon the symptoms. Three were eventually resuscitated by small doses of mannose, and one recovered slowly after a dose of fructose.

Lactose is incapable of giving any relief in mice to the symptoms produced by insulin. The results agree with those of Noble and Macleod in rabbits.

II. *Substituted Reducing Sugars.*

7. *Tetra-acetylfructose.* This sugar was available in a 5 % solution and was tested upon 10 mice, the total dosage in each mouse varying from 9 to 19 minims. The tetra-acetylfructose was ineffective in relieving symptoms in six cases, but appeared to produce a temporary alleviation, as indicated by a suspension of the convulsions, in three. One mouse, receiving a single dose of 17 minims, gradually recovered without further treatment, but it was one of a batch of three in which one had no symptoms, and the other, which had mild symptoms similar to those of the one injected, recovered without any treatment in much the same way. All the other nine were subsequently treated with glucose, whether temporary relief or no relief at all had resulted from the injections of tetra-acetylfructose.

In one mouse 16 minims of tetra-acetylfructose were injected. Temporary improvement followed the second dose, but it may not have been due to the action of the sugar injected, although the flexor spasms noted after its admini-

stration suggest that it had a beneficial effect. In other two mice, as already mentioned, the tetra-acetylfructose appeared to have a temporary beneficial effect. Fructose itself has been shown to be capable of alleviating symptoms but not of producing complete cure. The tetra-acetylfructose was only one-third of the strength of the fructose solution, and in the doses given could not be expected to have much effect. It is possible that the acetylation of fructose does not altogether render it incapable of action, but from the results obtained it is clear that this modified sugar is an ineffective one.

8. 2:3:5:6-*Tetramethylglucose.* An 11·4 % solution of this sugar was used in 11 mice. In none of these did any apparent result follow the injection. The sugar had no effect in relieving symptoms, whether slight or severe, neither did it seem to do any harm. All the mice were eventually treated with glucose and made complete recoveries.

9. 2:3:5-*Trimethylglucose.* A 10 % solution was employed in five mice. The symptoms varied from slight to severe. One mouse died after receiving 17 minims in three doses at intervals of 11 and 15 minutes between. In none did any apparent benefit ensue, and the four mice which survived were resuscitated by glucose, 4 minims in each case.

III. *Glucosidic Compounds.*

10. *Sucrose.* A 15 % solution was tested in seven mice. The dosage varied from 5 to 10 minims in each. Two of the mice died after receiving 5-minim doses without showing any improvement. One received 10 minims in two doses of 5 minims each, and died 11 minutes after the first dose, 5 minutes after the second dose. In the latter animal an injection of 5 minims maltose 3 minutes before death failed to elicit any improvement. The other four mice showed no improvement to two doses of sucrose in each case, and were finally resuscitated by maltose.

The results confirm the observations of Noble and Macleod in rabbits that sucrose is incapable of alleviating the symptoms due to insulin.

11 and 12. *α- and β-Methylglucoside.* A 10 % solution of α-methylglucoside was tested upon 11 mice, and a 10 % solution of β-methylglucoside upon two mice. Neither solution had any apparent effect upon the insulin symptoms, although used in a dosage up to 10 minims. One mouse died after an initial dose of 2 minims α-methylglucoside. The other 10, which showed symptoms of various degrees of severity, were not visibly affected in any way by repeated injections of larger doses, but each of them was eventually resuscitated by a small dose of glucose. Of the two mice injected with β-methylglucoside one showed no change to 4 minims of the solution and later required glucose. The other had slight symptoms only, which were not affected by 2 minims, and recovered without further treatment.

This compound is an important one, for in it the glucose molecule is altered by substitution in the least possible manner, but neither the α- nor the β-form showed the slightest sign of activity.

13. *Tetramethyl-β-methylglucoside.* This substance was used in a 10 % solution in six mice in doses of from 3 to 10 minims. In no case was there any alleviation of the symptoms noted. One mouse died, and the others were resuscitated by small doses of glucose.

The compound is inactive.

14. *Tetramethyl-γ-methylglucoside.* A 10 % solution was tested upon five mice in doses of from 5 to 10 minims. It had no appreciable effect upon the symptoms. One mouse, with slight symptoms only, recovered slowly after a dose of 5 minims. Another showed no change to 9 minims and recovered after two doses of fructose. The other three mice received doses of 10 minims each, and gradually becoming worse were resuscitated by small doses of glucose.

The compound is inactive.

15. *Glucose monoacetone.* A 12 % solution was tested upon four mice in doses of 8 to 10 minims. Two mice recovered gradually without further treatment, but their symptoms were slight and the injection made no apparent difference. The other two showed no change to 10 minims each, and gradually becoming worse were resuscitated by glucose. Glucose monoacetone is inactive.

16. *Salicin.* A solution of 14 % was employed, but had to be kept at body temperature and the syringe heated to prevent crystallisation at this strength. The solution was tested upon seven mice. The results obtained with this material are somewhat difficult of interpretation. The mice employed were small, 15 to 23 g., and not more than 3 minims of the solution was injected as a single dose. This amount was repeated once in each of three mice. Slight improvement appeared to result from the first dose in four instances, but none from the second dose. Two mice recovered slowly after a single dose of 3 minims, but as the other mice of the same batches also recovered without any treatment it cannot be inferred that their recovery resulted from the injection of salicin. The other five mice were all subsequently treated with glucose, but one which had received two doses of salicin without any improvement was in a moribund condition at the time it received glucose. It made a rapid recovery but died 10 minutes later with haemorrhage from the lungs. In all the mice which had severe symptoms a second dose of salicin was followed by an intensification of the symptoms, and the recovery resulting from 3 minims glucose was comparatively slow. The impression was formed that the salicin had actually done harm. The salicin was certainly not capable of effecting a complete recovery, and it is doubtful if it had any beneficial effect in temporarily alleviating symptoms.

IV. *Alcohols related to the Sugars.*

17. *Mannitol.* A 15 % solution was tested upon four mice. None of them showed the slightest improvement to doses of 3 minims. Two which had slight symptoms only recovered slowly after a single dose of 3 minims each, their

controls also recovered in the same way without any treatment. The other two mice were rapidly resuscitated by a single dose each of 2 minims glucose.

Mannitol is inactive.

18. *Dulcitol.* A 15 % solution was tested upon four mice in doses of 3 minims each, once repeated in two of them. No change in the severity of the symptoms was occasioned. Two mice, showing slight symptoms only, recovered slowly without further treatment, and the other two made a rapid and complete recovery to a dosage of 2 minims glucose.

Dulcitol is inactive.

V. *Anhydro-sugars.*

19. *β-Glucosan.* This material was tested in a 10 % solution upon three mice. One had two doses of 5 minims each with an interval of 10 minutes between. It showed no improvement but was rapidly relieved and permanently restored by a dose of 4 minims mannose. The second mouse had a total quantity of 14 minims injected at intervals of 13 and 6 minutes, and died 4 minutes after the last dose. The third mouse received in all 17 minims in three doses, showed no improvement, and was eventually resuscitated by mannose.

β-Glucosan is inactive.

A few experiments were made to ascertain if a mixture of two substances, one of them having a partial action, and the other no action when separately tested, was capable of exercising a greater influence upon the insulin symptoms. Equal parts of a 10 % solution of galactose and a 10 % solution of α-methyl-glucoside were mixed and used as the test because the results obtained from this mixture have some bearing upon the chemical problem. The experiments show that the mixture has no greater effect than that of the galactose alone.

Summary.

Of the unsubstituted sugars tested, glucose and mannose are found to be equally efficient in alleviating the symptoms and bringing about the complete recovery of mice suffering from insulin convulsions or coma. The hypodermic injection of from 2 to 5 minims of a 15 % solution of either of these sugars suffices in most instances, though its repetition is sometimes necessary.

Fructose is much less effective than glucose or mannose. It usually induces a temporary alleviation of the symptoms, more especially of the convulsive stage, and, if the dose of insulin has not been too large, the injection of fructose may enable the animal to recover. Relapses are more frequent after the use of fructose, larger doses are required, and there is not the same rapid relief; even after the convulsive stage is recovered from, weakness of the muscles persists after fructose, which is not the case when glucose or mannose is employed. The conclusion is reached, confirmatory of Noble and Macleod in rabbits, that fructose cannot take the place of glucose.

Galactose has an action similar to that of fructose, but is even less efficient. It does not always produce alleviation of the symptoms and when it does

effect a cessation of the convulsions, such relief is of very short duration. Galactose is incapable of effecting a complete cure, and cannot take the place of glucose. (Confirmatory of Noble and Macleod.)

Maltose is an active sugar, but is inferior to glucose and mannose. It can relieve symptoms and effect a permanent cure, but is somewhat slower in action, and the dosage required is larger than that of glucose or mannose. With respect to this sugar its action on mice appears to be much more efficient than was found to be the case by Noble and Macleod in rabbits.

Lactose is inactive. (Confirmatory of Noble and Macleod.)

Three substituted reducing sugars have been tested. Of these tetra-acetylfructose alone showed signs of activity. It appeared to produce a temporary alleviation of convulsions in a few instances, but its action in this respect is doubtful. Tetra-acetylfructose is incapable of effecting recovery from insulin symptoms. The other two compounds tested, tetramethyl-glucose and trimethylglucose, are devoid of activity.

Seven glucosidic compounds were tested. Sucrose, α- and β-methyl-glucoside, tetramethyl-β-methylglucoside, tetramethyl-γ-methylglucoside, and glucose monoacetone are inactive. The results obtained from salicin suggest that this compound may actually do harm. It alone of the glucosidic compounds gave any indication of being able to exercise an action. It is possible that salicin undergoes hydrolysis in the body; a slight beneficial action was sometimes apparent after a first dose, but none after a second. The compound is incapable of effecting a cure of the insulin condition.

Two alcohols related to the sugars were tested, mannitol and dulcitol. Neither alcohol had any apparent activity.

One anhydro-sugar, β-glucosan, was tested and found to be inactive.

None of the substances tested appeared to have any deleterious action with the possible exception of salicin.

A mixture of equal parts of galactose and α-methylglucoside was tested to ascertain if the partial action of galactose is enhanced by the presence of a glucose molecule in which the reducing group is the only one substituted. The action of the mixture was found to be no greater than that of the galactose alone.

PART II.

Discussion of Results in Terms of Carbohydrate Structure.

The sugars and related compounds, the effects of which were studied at St Andrews, may be classified as under:

I. Unsubstituted Reducing Sugars.

Glucose. This was used chiefly to establish the standard against which other results might be compared.

Mannose, Fructose, Galactose, Maltose, Lactose. The use of the above compounds determined the effect of the reducing group in conjunction with different asymmetric systems.

II. Substituted Reducing Sugars.

Tetra-acetylfructose, 2:3:5:6-*Tetramethylglucose*, 2:3:5-*Trimethylglucose*. The above compounds were employed so as to ascertain the effect of the reducing group alone.

III. Glucosidic Compounds.

Sucrose, *α-Methylglucoside*, *β-Methylglucoside*, *Tetramethyl-β-methylglucoside*, *Tetramethyl-γ-methylglucoside*, *Glucose monoacetone*, *Salicin*.

IV. Alcohols related to the Sugars.

Mannitol, *Dulcitol*.

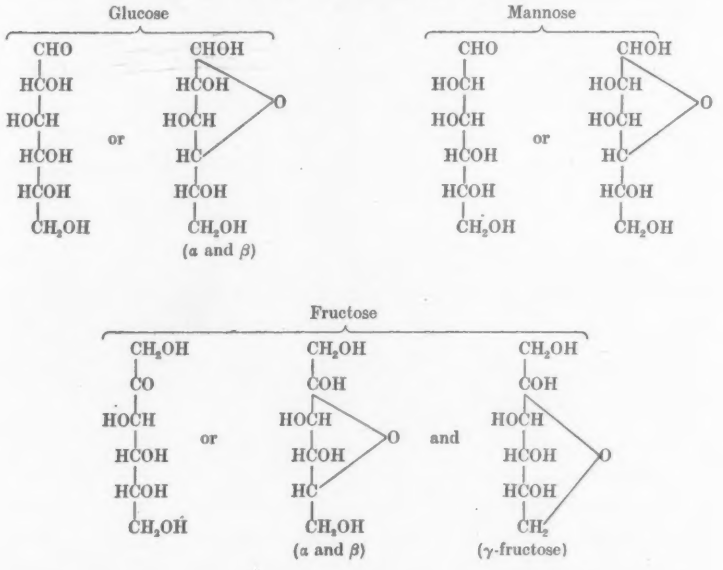
V. Anhydro-Sugar.

β-Glucosan.

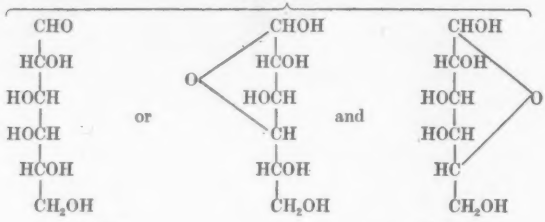
The compounds classified in Sections III, IV and V were employed to test the effect of eliminating the reducing group in different ways, six varieties being introduced.

In order to make the discussion intelligible the structural formulae assigned to each of the test substances employed are now given:

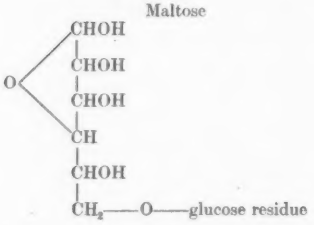
I. *Unsubstituted Reducing Sugars.*



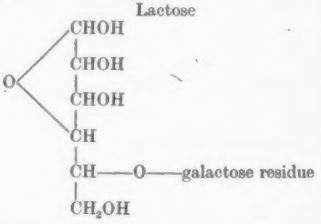
Galactose



Maltose

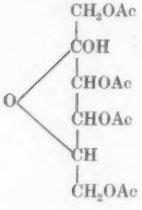


Lactose

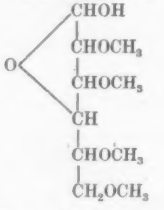


II. *Substituted Reducing Sugars.*

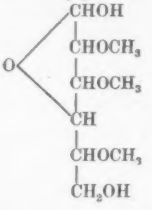
Tetra-acetyl fructose



2 : 3 : 5 : 6-Tetramethylglucose

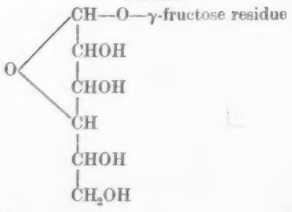


2 : 3 : 5-Trimethylglucose

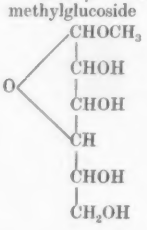


III. *Glucosidic Compounds.*

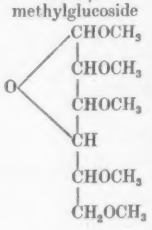
Sucrose



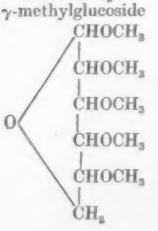
α- or β-methylglucoside



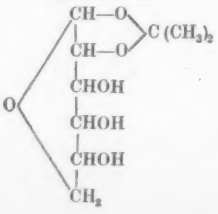
Tetramethyl α- or β-methylglucoside



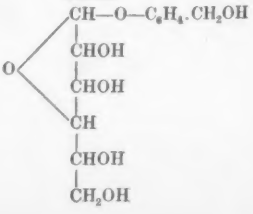
Tetramethyl γ-methylglucoside



Glucose monoacetone

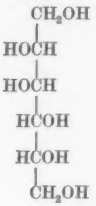


Salicin

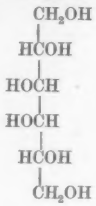


IV. *Alcohols related to the Sugars.*

Mannitol

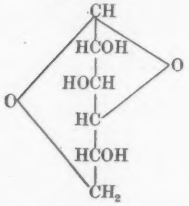


Dulcitol



V. *Anhydro-sugar.*

β-Glucosan



Another method of displaying the nature of the test compounds is to refer them by means of a skeleton formula to the glucose molecule, and indicate by a numerical method the positions which are substituted or modified in each of the derivatives used. If the carbon atoms of the glucose molecule be indexed by the numbers 1 to 6 as below:

C ..... 1 (reducing group)
|
C 2
|
C 3
|
C 4
|
C 5
|
C ..... 6 (primary alcohol group)

then the following table shows how the glucose molecule has been varied in each test compound employed:

Position 1 substituted	α- and β-Methylglucosides Sucrose Salicin
Position 2 modified	Mannose
Position 4 modified	Galactose
Position 5 substituted	Lactose
Position 6 substituted	Maltose
Positions 1 and 2 modified	Fructose; mannitol
Positions 1 and 2 substituted	Glucose monoacetone
Positions 1 and 4 modified	Dulcitol
Positions 1 and 6 substituted	β-Glucosan
Positions 2, 3, 5 substituted	Trimethylglucose
Positions 1, 3, 4, 6 substituted and 2 modified	Tetra-acetylfructose
Positions 2, 3, 5, 6 substituted	Tetramethylglucose
Positions 1, 2, 3, 5, 6 substituted	Tetramethyl-β-methylglucoside
Positions 1, 2, 3, 4, 5 substituted	Tetramethyl-γ-methylglucoside

The twenty test compounds thus represent fourteen structurally defined variations in the glucose molecule. It is important to remark that the purity of the materials was the highest obtainable, the actual specimens employed being those upon which the physical constants have been standardised. This remark has special significance in the case of maltose, average specimens of which are notoriously variable in composition, the impurities being generally dextrins, isomaltose and free glucose.

The effects of the various sugars may be classified as under, the compounds being arranged in order of efficiency:

Class I.

Complete positive results, *i.e.* abolition of irritability and convulsions, rapidly followed by complete and permanent recovery.

Glucose
Mannose } practically equal in effect.

Class II.

Complete positive results as above, but more slowly produced.

Maltose.

Class III.

Partial positive results, *i.e.* immediate abolition of irritability and convulsions, followed very soon by recurrence of the same symptoms; no permanent recovery.

Fructose.
Galactose.
Tetra-acetylfructose.

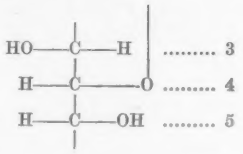
Class IV.

Entirely negative results, but with the possible exception of salicin, no toxic symptoms.

All the remaining compounds mentioned in the list.

Taking the foregoing results in conjunction with the facts already known that arabinose, xylose, glycerol and the mixture of glyceric aldehyde and dihydroxyacetone are without effect, a number of generalisations are justified. Of these, the most obvious is that the presence of a reducing group is an essential factor. This is shown by the entirely negative results obtained with all non-reducing compounds, and in this connection the use of α- and β-methylglucosides is instructive. In these compounds the reducing group of the parent glucose is modified to the minimum extent possible, yet the specific action of the glucose is eliminated entirely. Moreover, it is important to notice that the combined evidence points strongly to the idea that the functional sugars react not as aldehydes but as cyclic structures. If this were not the case, arabinose should behave similarly to glucose.

The second conclusion is that certain asymmetric systems of the sugar chain are necessary before a positive result is obtained and that others are not. Thus, whereas in glucose position 2 is H—C(OH)— the reverse arrangement HO—C(H)— is present in mannose yet the sugars function equally. Considering the profound effect on the reactions of sugars occasioned by alteration in the stereochemical position of the hydroxyl groups, it is highly improbable that the above system is operative in the curative reaction. It would also appear that the primary alcohol group is not involved, as although position 6 is unsubstituted in the majority of the test substances it is definitely replaced in maltose where the effect is positive. Positions 2 and 6 can thus be eliminated from the discussion, which is now focussed on 3, 4 and 5. In glucose these systems are formulated as under:

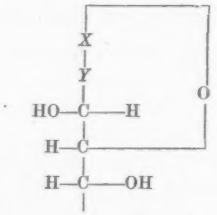


and the arrangement also exists in mannose and maltose. There is no direct evidence bearing on the function of 3, but 5 is of evident importance. Substitution of 5, as is the case with lactose, or ring-formation through this position as in arabinose destroys the curative action. Incidentally it may be remarked that this position is involved in the ring structure of the molecules of cellulose, of the amylo-pectin constituent of starch, and of glycogen. It is of course possible that the effect of maltose may be explained by hydrolysis to glucose as a result of enzyme action. The specimen used was free from either glucose or soluble dextrins yet a very decided positive result was obtained, although somewhat more slowly than by glucose or mannose. This suggests that the disaccharide was undergoing hydrolysis. It is, of course, impossible to verify or refute this suggestion, but as maltose is 6-glucosido-glucose it is evident that this particular sugar is the only reducing disaccharide in which the second hexose residue is not attached directly to any of the asymmetric carbon atoms. Irrespective of its mechanism, the result actually obtained is, therefore, consistent with the generalisation.

With regard to position 4 it may be noted that displacement of the oxygen ring has a pronounced effect. When the ring is formed on the opposite side of the chain of carbon atoms (e.g. one form of galactose) the positive reaction is diminished. Under some circumstances galactose may react in another form in which the ring is displaced to position 5, thus substituting a group which is evidently essential. It is important to emphasise that the presence of a reducing group alone is insufficient and the asymmetric systems of positions 3, 4 and 5 are ineffective in the absence of a reducing group. This is shown by

the negative behaviour of arabinose, xylose and lactose, all of which are reducing aldoses, but do not possess the complete asymmetric systems referred to. A more striking case leading to the same conclusion is presented by the tetra- and tri-methylglucoses. Here, although in each case the reducing group is free and the glucose configuration is preserved, the compounds are ineffective as the substituting methyl groups are stable and permanently mask the hydroxyl groups. Further, in both mannitol and sucrose the necessary asymmetric system is present, but there is no reducing group and the action is negative. The point now under discussion is best emphasised by the observation that a mixture of galactose (which contains a normal reducing group) and methyl glucoside (where the necessary asymmetry is present) was ineffective. Both conditions must therefore be satisfied in one molecule.

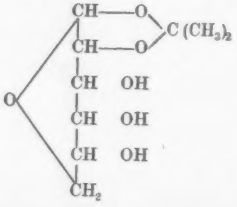
A review of the combined results shows no exception, which cannot be adequately explained, to the generalisation expressed in the statement that *the type of carbohydrate molecule functional in eliminating the convulsion symptoms occasioned by the administration of insulin is:*



where either X or Y represents a reducing group.

The asymmetric system formulated above will, of course, be affected to some extent by the nature of *X* and *Y*, and this suggests that *X* and *Y* may undergo rearrangement to give a common form.

It may further be claimed that if the above general formula be substituted in any respect, a necessary condition for the development of a positive result is that the substituting groups are removable either by enzymes or by dilute alkali. The case of glucose monoacetone is specially instructive as this compound has recently been proved to be a derivative of γ-glucose probably possessing the structure

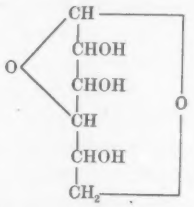


Despite the fact that the compound is hydrolysed with extreme ease through the agency of very dilute acids, being in this respect one of the most unstable

organic compounds so far known, it is devoid of effect. This may be taken as a test case proving that glucosidic derivatives of γ-glucose play no part in the changes now under discussion. The behaviour of sucrose shows that derivatives of γ-fructose can also be ruled out.

It is at this stage premature to offer any suggestions as to the chemical reactions which accompany or account for a positive result, but a review of all the evidence points to the idea that two series of reactions are involved and that they proceed consecutively or take place simultaneously at different speeds. Consideration of the curious behaviour of galactose affords some support to this view and indicates that the first reaction is an attack on the reducing group of the sugar, thus making possible a further change in which the asymmetric chain of CHOH groups participates. This opinion is also supported by the general experience that specific reactions of sugars take place in definite steps the first of which involves the reducing group, either alone or in conjunction with one other hydroxyl group. The remaining hydroxyl groups then react singly or in pairs according to their stereochemical position. In other words it is not the sugar as a whole which reacts but definite sections of the molecule.

Although the generalisations now put forward seem to exclude from the list of functional compounds the anhydro-glucoses, it was nevertheless deemed advisable to conduct experiments designed to test the effect of such molecules. The question at once arises if glycogen can replace glucose as a corrective carbohydrate, but the solubilities of the polysaccharide are inconvenient for this purpose. As an alternative, the simplest possible molecule possessing the structure $C_6H_{10}O_5$ was selected for trial and β-glucosan



was therefore employed. This compound failed to show any positive effect in relieving the symptoms produced by insulin, and the reasonable deduction is that glycogen as such plays no part as a corrective carbohydrate and can do so only if it undergoes hydrolysis to give glucose.

PART III.

Comments.

Although my participation in the foregoing experiments has been limited to a few preliminary observations in conjunction with the other two authors, I am requested by them to add a few comments, particularly with regard to the physiological aspects of the work. In so far as these relate to the same

sugars there is a general agreement with the results obtained by Noble and myself, such differences as exist being possibly accounted for by the fact that animals of different species were used in the two series of observations. In both of these, glucose and mannose were found to be by far the most efficient antidotes. In the experiments on rabbits, next in order, and of about equal efficiency, came maltose, fructose and galactose, but in those on mice, maltose was found to be decidedly more efficient than the other two sugars of this group. In the experiments on mice this sugar appeared to be almost as effective an antidote as glucose and mannose, whereas in those on rabbits it was decidedly less so. A conspicuous feature of the action of maltose in the experiments on rabbits was that temporary recovery often occurred shortly after the injections, followed by relapses in which the symptoms were either convulsions alone or convulsions, coma and ultimate respiratory failure. In some cases also the symptoms ended in death, although the blood sugar (as measured by the reducing power) had risen to over twice the normal level. It is possible that maltose cannot act as an antidote until after it has become hydrolysed to glucose and, if this be the case, inequality in the rates of this process in mice and rabbits may account for the difference in the results obtained in the two groups of experiments. It has not been possible to find in the literature any very definite information bearing on this aspect of the question. To judge from the results published by Bial [1893], to whose and to Hamburger's [1895] researches we owe the demonstration that maltase does actually exist in blood, there is no distinct difference in the activity of this enzyme in the blood of the ox, as compared with that of the dog or man, but it is unknown whether the blood of the mouse is of the same potency as that of the rabbit.

Since the conclusion, that the primary alcohol group of the glucose molecule is not essential, depends on the positive results obtained with maltose, N. B. Taylor, at my request, has performed a few experiments comparing the antidoting effects of maltose (commercial) and glucose on the hypoglycaemic symptoms in cats and dogs; and we have also repeated them on rabbits. In a cat showing the hypoglycaemic symptoms characteristic for this animal (profuse salivation, ataxia, pupillary dilatation and mewing followed by a semi-comatose state with muscular twitching and inability to stand), 2·4 g. per kilo. body weight of maltose caused no visible improvement in 15 minutes, but in 35 minutes the pupils were seen to be less dilated and the animal was able to stand. It was not until about 1 hour after administration of the maltose, however, that recovery could be considered in any way complete and the animal was able immediately to gain its equilibrium when dropped from a height of 4 feet. In the same cat, two days later, injection of 1·2 g. of glucose per kilo. body weight was followed by a similar degree of recovery in 16 minutes although the hypoglycaemic symptoms were more marked (deep coma). In another cat in which the most prominent of the symptoms were convulsions, 1·3 g. of maltose (per kilo.) brought about a corresponding degree of recovery in 30 minutes. In a third animal, in deep coma which had been

preceded by convulsive symptoms, 0·6 g. of maltose (per kilo.) caused a doubtful improvement in 20 minutes but this did not become more evident as time went on so that another similar amount was injected in 45 minutes. One hour after this second injection consciousness had returned but the animal was unable to stand.

The conclusion is that maltose is usually decidedly inferior to glucose in removing the symptoms of hypoglycaemia caused by insulin in the cat.

In a dog in which symptoms of moderately deep coma had existed for a little over one hour, 0·6 g. maltose per kilo. had absolutely no effect in 20 minutes, after which the same dose was repeated. Although the symptoms became somewhat less marked, another injection of 1·2 g. per kilo. was given; after 10 minutes there was some improvement and in 35 minutes the animal sat up although it could not stand erect. This improvement did not last for long, however, for 10 minutes later the coma had again become much more intense, and the animal unable to hold up the head. The condition did not improve during the next 90 minutes, after which 0·6 g. glucose was injected, followed by 1·2 g. 10 minutes later, with the result that the animal was able to stand and to walk a few steps in 20 minutes after the last injection.

In this case there is no doubt that maltose was inferior to glucose as a restorative.

In another dog in which convulsions were a prominent feature of the symptoms, although coma was also prominent, 0·6 g. of glucose brought about decided improvement in 3 minutes (convulsions disappeared and animal sat up) and consciousness very quickly returned, so that the animal was practically normal within 7 minutes of the injection of glucose. This did not last for long, however, for in 22 minutes after the first sugar injection the coma had again become profound, indicating a relapse of the hypoglycaemic symptoms due no doubt to the using up of all the previously injected glucose, so that a further 0·6 g. glucose was injected. Since no improvement was observed after 13 minutes a further quantity of 1·2 g. per kilo. was injected. This caused decided improvement within 7 minutes and thereafter the animal gradually improved and became normal in 37 minutes after the second injection.

In numerous other observations in this laboratory in which glucose has been used to remove the hypoglycaemic symptoms in dogs it has been noted that relapses are liable to occur and it is our practice to make the injections at intervals. There can be no doubt, however, that maltose is decidedly inferior to glucose as an antidote in this animal.

In three rabbits exhibiting convulsions recently injected with maltose one recovered as promptly as would have been the case with glucose and did not relapse, but in two, although there was prompt improvement, the convulsions returned within half an hour. One of these animals had ultimately to be given glucose to restore it.

As Irvine points out, it is impossible to say whether or not hydrolysis to glucose must precede the antidoting action of maltose. It seemed to act more

efficiently in the experiments on mice than in those on larger animals but it must be pointed out that the maltose used in Toronto (Schuchardt's), unlike that in St Andrews, was not chemically pure and may therefore have contained considerable quantities of dextrin which would be very slowly, if at all, hydrolysed in the blood.

It is of interest to compare the results of these two groups of observations on the hypoglycaemia due to insulin with those obtained by Mann and Magath [1921, 1922] on that following removal of the liver from dogs. These observers found that the hypoglycaemic symptoms were rapidly removed by the injection of 0·25 to 0·5 g. glucose per kilo. body weight and that a certain benefit was also obtained by the injection of maltose, mannose, dextrin and galactose. With regard to maltose it was found that when first injected its influence in removing the symptoms was only slowly displayed, whereas the second injection acted much more quickly. This may have some relationship to the maltase content of the blood. Mannose acted with about the same efficiency as maltose, which would place it at a lower value than we have judged it to be. The effect of dextrin is described as being very slowly developed and slight in degree, and of galactose as being even less definite than that of dextrin. Curiously enough fructose was found to have no restorative effect. With regard to other particulars, the results obtained by Mann and Magath agree exactly with those obtained in the experiments in which insulin was used.

It is significant that the sugars which act best as antidotes for the hypoglycaemic symptoms are those that are directly fermentable by yeast: glucose, fructose and mannose. The interesting point also comes to light that galactose, which we have seen to be so decidedly inferior as a restorative in insulin hypoglycaemia, is also much less fermentable than any of the other three hexoses [E. F. Armstrong, 1905]. It seems to be agreed that glucose and fructose are fermented at the same rate both by normal yeasts and by yeasts the action of which has been crippled in various ways, whereas mannose is fermented at varying rates according to the variety and the condition of the yeasts. On the other hand, the antidoting value of mannose in hypoglycaemia is at least as great as that of fructose.

REFERENCES.

Armstrong (1905). *Proc. Roy. Soc. London*, B, **76**, 600.
Bial (1893). *Pflüger's Arch.* **53**, 156; **54**, 72.
Hamburger (1895). *Pflüger's Arch.* **60**, 543.
Mann and Magath (1921). *Amer. J. Physiol.* **55**, 286.
—— —— (1922). *Arch. Int. Med.* **30**, 73, 171.
Noble and Macleod (1923). *Amer. J. Physiol.* **64**, No. 3.

CXXXVI. ON THE BIOCHEMISTRY OF SKIN, AND THE CHEMICAL BASIS OF SKIN SWELLING.

By MADGE KAYE and DOROTHY JORDAN LLOYD.

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I. On the Biochemistry of Skin.

In previous investigations by the authors [1924] it has been shown that the swelling of a fibrous tissue, such as skin, depends on both structural and chemical factors. The chemical factors considered were those due to variation in the external swelling medium. There remain those contributed by the skin itself. The influence of these is well shown by comparing the swelling of fresh goat skin with that of commercial sun-dried goat skin. The drying of the skin at a tropical temperature has caused a chemical change in its proteins which is made visible by the firm cementing together of the fibrils of the fibres. Skin is composed chiefly of proteins, though it contains in addition fats, sterols, and sterol esters. Rosenthal [1916] gives the following analysis of calf skin dried *in vacuo* at 55–60°.

Fresh calf skin	Butt	Shoulder
Albumin and globulin	4·14	5·16
Mucoids	4·81	2·29
Elastin	12·31	16·74
Collagen	58·83	39·66
Keratin, etc.	19·91	36·15
Total dry weight	100·00	100·00

The different proteins are distributed in the skin as follows: the keratin in the hair and epidermis; the elastin in the elastin fibre network of the grain; the collagen in the skin fibres, and the albumin and globulin, as will be shown later, although possibly present to some extent in the skin fibres, chiefly in the interfibrillary matrix. The evidence for the localisation of the last two is indirect, and is derived mainly from the parallel behaviour exhibited by plasma proteins (albumin and globulins) and by "interfibrillary substance" under the same external conditions. Albumins and globulins differ from the other classes of skin protein by being irreversibly coagulated by heat. Heat coagulated serum albumin is insoluble in water, and dried serum always contains an insoluble residue if the temperature of drying be allowed to rise above 37° [Michaelis and Rona, 1910; Hartley, Eagleton and Okell, 1923]. Eitner [1880] has shown that skins dried at a series of temperatures become increasingly difficult to soften in water in proportion to the temperature at which they are dried.

In the paper previously quoted [1924] it has been shown that in sun-dried skins the fibrils are cemented together by some non-elastic material. This condition is not found in fresh skin, and the material can be removed by ammonia, trypsin, and strong solutions of sodium chloride. In these properties, therefore, it behaves like a coagulated albumin.

Additional evidence bearing on the nature of the interfibrillary substance is obtained by a further comparison of the behaviour of fresh and sun-dried skins. The swelling curve of fresh goat skin over a wide range of reaction is shown in Fig. I (*a*). It is of the same type as that for sun-dried skin shown

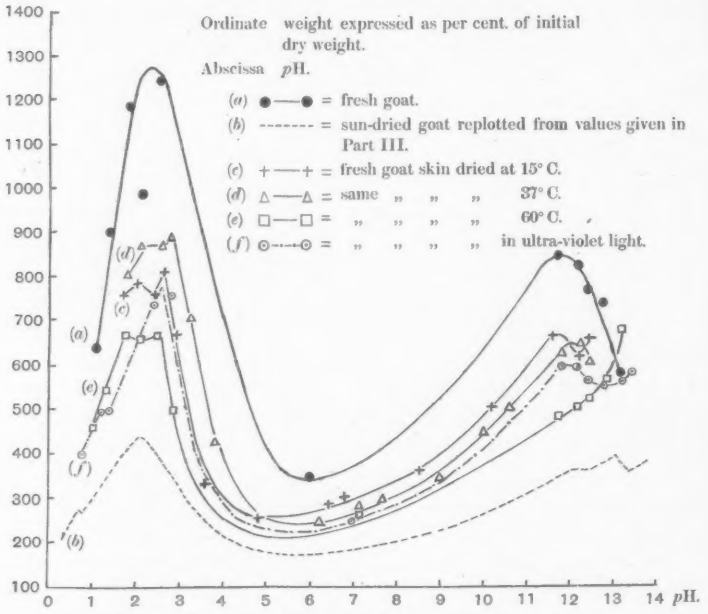
Ordinate weight expressed as per cent. of initial dry weight.
Abscissa pH.
(a) ●—● = fresh goat.
(b) ------- = sun-dried goat replotted from values given in Part III.
(c) +—+ = fresh goat skin dried at 15° C.
(d) △—△ = same " " " 37° C.
(e) □—□ = " " " " 60° C.
(f) ⊙····⊙ = " " " " in ultra-violet light.
1400
1300
1200
1100
1000
900
800
700
600
500
400
300
200
100
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 pH.

Fig. 1. The effect of albumin-coagulation on skin swelling.

in the same figure, curve (*b*), but differs from it in its much higher level throughout, particularly at the acid maximum. In the dried skin the acid and alkali maxima lie at about the same level; in fresh skin the acid maximum lies, as in swollen gelatin, well above that attained with alkali. Besides this difference in the quantitative behaviour, certain qualitative differences are also found between fresh and dried skin; for instance, in the lower concentrations of the sodium hydroxide the fresh skin pieces are thick, white, and flaccid, and a microscopical examination shows that extensive splitting of the fibres has taken place. This condition has never been found in sun-dried skin treated with similar solutions. Tables I and II, which give the external and internal condition of soaked skin at most of the experimental points recorded on

curves (*a*) and (*b*) in Fig. 1, are given below. It can be seen that in the fresh skin splitting of the fibres is found throughout the series whilst in the dried skin there is no splitting except in the strongly acid solutions. Experiments made on sun-dried goat skin by treating it with trypsin, showed that by this means the skin could be brought nearer to the fresh condition. The swelling curve for a skin so treated is shown in Fig. 2, curve (*c*) and its structural condition described in Table III. It will be seen that the fibrils have been separated by the trypsin and the samples absorb water more freely throughout the series. At the lower concentrations of alkali there occurred the separated condition of the fibrils approaching that of fresh skin at these points.

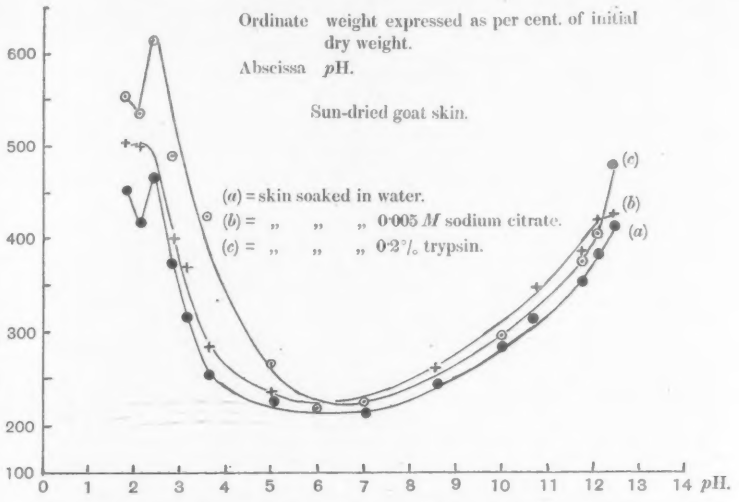
Ordinate weight expressed as per cent. of initial dry weight.
Abscissa pH.
Sun-dried goat skin.
(a) = skin soaked in water.
(b) = „ „ „ 0·005 M sodium citrate.
(c) = „ „ „ 0·2% trypsin.
600
500
400
300
200
100
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 pH.

Fig. 2. The effect of dispersing coagulated albumin on skin swelling.

In the fresh skin the material between the fibrils must be in a fluid condition, since it offers no resistance either to the swelling or to the separation of the fibrils. In the dried skin, apparently this material between the fibres has passed into an insoluble condition, cements the fibrils together and causes mechanical interference with swelling. These facts are consistent with the view that the fibrils are surrounded by interfibrillary proteins of the same nature as the plasma proteins.

Albumins are irreversibly coagulated not only by heat but also by light [Young, 1922], though Harris [1923] states that this only occurs "after prolonged exposure." Coagulation also occurs when albumins are dried by means of alcohol at room temperature, but not at zero centigrade [Hardy and Gardiner, 1910]. In order therefore to prove that coagulating agents acting on a skin during drying lead to a cementing together of the fibrils, the following experiments were tried: the skin of a freshly killed goat was taken and cut

into small pieces (about 3 × 4 inches). The samples were dried in various ways (see Table IV) and were soaked for three days, (*a*) in water, or (*b*) in a 0·2 % solution of commercial trypsin.

Table I. *Fresh goat skin. Swelling in acid and alkali.*

No. of sample	p_H at equilibrium	Macroscopical examination	Microscopical examination
1	1·8	Thick, swollen, semi-transparent and rubbery	Fibrils separated and very well swollen; a few encircling bands remain
2	2·4	Thick, swollen, but not so transparent and rubbery as before	Fibrils separated and well swollen. Only a few bands remain
3	2·7	Thick, swollen, and fairly rubbery but opaque	Fibrils swollen. Many bands remain. Where bands are broken the fibrils are separated
4	4·4	Fairly thick and swollen but rather soft and white	Fibrils fairly swollen but mostly confined into bundles. Some bands are ruptured
5	7·6	Thin, flaccid and opaque	Fibrils thin, unswollen and wavy; no encircling bands evident
6	9·0	A little thicker but soft, flaccid and opaque	Fibrils unswollen but well separated
7	10·1	As thick as No. 6. Quite soft, flaccid and opaque	Fibrils similar to No. 6, but slightly thicker
8	12·0	Much thicker and more swollen and rather rubbery. Still rather soft and white	Fibrils a little more swollen and well separated
9	12·3	Thick, swollen, semi-transparent and rubbery	Fibrils much thicker and fairly well separated. No encircling bands

Table II. *Sun-dried goat skin. Swelling in acid and alkali.*

No. of sample	p_H at equilibrium	Macroscopical examination	Microscopical examination
1	1·9	Thick, swollen and rubbery	Fibres swollen but not separated into fibrils
2	2·3	Very thick, swollen and rubbery	Fibres swollen but not separated
3	3·4	Thin, only slightly swollen	Fibres only slightly swollen
4	5·9	Thin, not swollen	Fibres neither swollen nor separated
5	6·9	Thin, not swollen	Fibres thin; neither swollen nor separated
6	9·2	A little thicker but very soft	Fibres thin but slightly separated
7	11·0	Thin and soft	Fibres thin and only slightly separated
8	11·4	Thicker and partially swollen but still soft	Fibres partially swollen and slightly separated
9	12·2	Swollen, fairly thick and stiff; rubbery	Fibres swollen but not separated
10	12·5	Fairly thick; swollen and stiff; rubbery	Fibres swollen but not separated
11	12·8	Thick, swollen, very stiff and rubbery	Fibres swollen but not separated

Table III. *Sun-dried goat skin soaked in pancreatin. Swelling in acid and alkali.*

No. of sample	p_H at equilibrium	Macroscopical examination	Microscopical examination
1	2·3	Very swollen, thick and rubbery	Fibrils well swollen and separated
2	2·6	Very swollen, thick and rubbery	Fibrils swollen and separated
3	3·2	Fairly thick, swollen and rubbery but rather opaque	Fibrils only partially swollen but very well separated
4	4·9	Thin, not swollen, but opaque	Fibrils partially separated
5	7·0	Thin, not swollen but white, opaque and flaccid	Fibrils separated but not swollen
6	8·3	Thin, flaccid and not swollen	Fibrils only partially separated; not swollen
7	9·2	Thin, flaccid and not swollen	Fibrils thin and partially separated
8	10·5	A little thicker but soft and flaccid	Fibrils slightly swollen and partially separated
9	12·0	Fairly swollen but still rather opaque	Fibrils a little more swollen and separated
10	12·3	Fairly thick and swollen. Rubbery	Fibrils fairly swollen and separated

Table IV. *Results.*

No.	Experimental conditions	Series A. Water soak		Series B. 0·2 % Trypsin soak	
		Macroscopical examination	Microscopical examination	Macroscopical examination	Microscopical examination
1	Fresh goat. Undried control	Very supple and thick	Well split into fine fibrils	Very supple and thick	Rather more split than 1 A. Transparent
2	Dried at 15° C in dull light	Supple but slight feeling of hardness	—	Very soft but not quite so thick as B 1	—
3	Dried at 40° C. in dull light	Slightly harder than 2 A	Fibres not split into fibrils	,,	Fibres well split into fibrils
4	Dried at 60° C. in dull light	Marked hardness; very thin	,,	,,	,,
5	Dried at 80° C. in dull light	Horny and contracted	—	Contracted and rotten	—
6	Dipped for 30 secs. in boiling water	Contracted and rubbery	Not split into fibrils. Fibres are shrunken	Contracted. Slimy on the flesh side	Fibres swollen, transparent and broken into short pieces
7	Dried under mercury arc	Very hard	No separation into fibrils	Soft	Well separated into fibrils
8	Dried in absolute alcohol at 15° C.	Rather hard	Fair splitting into fibrils	Very soft	Well split
9	Dried in absolute alcohol at 0° C.	Very soft	Well split	Very soft	Well split

Note. The B series throughout is whiter than the A series, showing that there is a greater separation into fibrils.

It can be seen that in every experimental condition which is such as would cause irreversible coagulation of serum albumin, the skin becomes difficult to soak, and the hardness is proved by microscopical examination to be due to the non-separation of the fibrils. The absolute identity of the conditions producing hardness with those causing irreversible coagulation shows that the interfibrillary substance must be of the nature of protein and that the cement-

ing of the fibrils found in some dried skins must be due to its coagulation. The effect of trypsin in removing the interfibrillary cement confirms this view. Conditions of extreme coagulation lead to destruction of the fibrils, suggesting that albumin may also be present to some extent in the fibrils themselves.

It is to be expected that any reagents which destroy or dissolve coagulated albumin should lead to a separation of the fibrils and to an increased power of water absorption by the skin as a whole. Among such substances are: trypsin, neutral solutions of salts, acids, and alkalies.

1. *Trypsin* rapidly digests coagulated albumin. Its effectiveness as an agent for separating fibrils, and its influence on water absorption has been previously described [Kaye and Jordan Lloyd, 1924].

2. *Neutral solutions of salts* disperse coagulated serum albumin, the efficiency of the salt varying with the basicity of the acid radicle, as shown by Chick and Martin [1910, 1911, 1912, 1, 2]. The most efficient salts are those which form ter- or quadri-valent negative ions in solution, among which Chick and Martin specially mention sodium citrate. Experiments were made to test the effect of sodium citrate, sodium pyrophosphate, and sodium ferrocyanide on sun-dried skins. The results are given in Table V. It can be seen that in every case the salts have the power of removing interfibrillary substances at the same concentration as would disperse coagulated albumin.

Table V.

No.	Soak-liquor	Macroscopical examination	Microscopical examination
1	Distilled water	Rather horny	Only coarse fibres showing
2	Sodium citrate 0·005 *M*	Softer than 1	Well separated into fibrils
3	Sodium ferrocyanide 0·005 *M*	About like 2	,, ,,
4	Sodium pyrophosphate 0·005 *M*	Soft and soaked	,, ,,
5	Trypsin 0·2 % solution	Very soft and white	Split into fine fibrils

3. *Acids and alkalies.* Both these have the power of dispersing heat-coagulated albumin, but they also have the property of causing swelling of the fibrils. For this reason, when sun-dried skin is soaked in dilute solutions of hydrochloric acid or sodium hydroxide, the fibrils distend very rapidly and close up the interfibrillary spaces, thus checking any chemical action which might take place in these spaces. Acids and alkalies in general, therefore, although they disperse coagulated albumin, do not loosen the skin structure except under a few special circumstances.

Ammonia stands out from most alkalies as being one of the most effective substances known for separating the fibrils. This is probably connected with its weak ionisation and with its special power of penetrating cell walls.

The analogy between the behaviour of serum albumin and "interfibrillary substance" is thus seen to be a very complete one; both are rendered insoluble (coagulated) by the same conditions, and both can be again taken back into solution by the same means. The influence of coagulating factors (heat, ultra-

violet light and alcohol) acting during the drying of skin, on subsequent water absorption, is shown graphically in Figs. 1 and 4. In every case the water absorption has been reduced below the level of that of fresh skin. The influence of dispersing agents, such as trypsin or sodium citrate, on skins in which coagulated albumin may now be presumed to occur, is shown in Figs. 2, 3 and 4. In Fig. 2 it can be seen that the water absorption of a commercial dried skin can be increased by previously soaking for three days with 0·005 *M* sodium citrate or 0·2 % trypsin. In Fig. 3 is shown the influence of heat in reducing the power of water absorption, the influence of trypsin in restoring

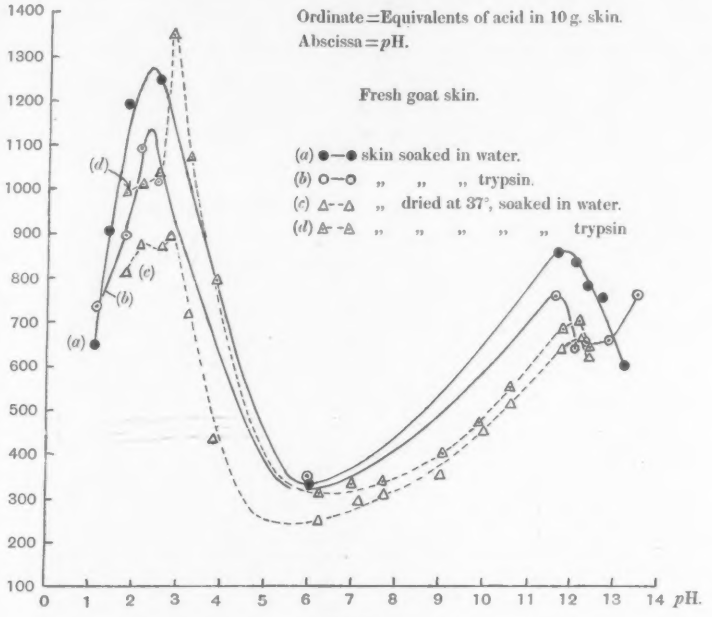

Fig. 3.

it. It must be borne in mind that drying *per se* has an influence on the subsequent behaviour of the skin. Maclaughlin and Theis [1923] have shown that mere withdrawal of water from a skin has, in itself, an action on the skin which checks re-absorption. They showed that samples of skin (ox) dried to varying extents (up to half the total water) at a constant temperature of 33°, when immersed in either water or saturated lime water (p_H 12·4) re-absorbed water in proportion to the water remaining in the partially dried skin. The temperature at which they dried their material should have precluded the possibility of heat coagulation. It can also be seen from Fig. 1 that drying a skin at a temperature as low as 15°, lowers the capacity for absorbing water at all reactions. The skin so dried and then soaked in water is not so supple as

fresh skin. The crucial test as to the influence of drying as apart from coagulation is found in the experiments on drying with alcohol at 0°. This method was used by Hardy and Gardiner [1910] to prepare pure serum proteins in a chemically unchanged condition. Skins dried by this method become as soft as fresh skins when soaked in water, but in spite of this their swelling power has been reduced. This is shown graphically in Fig. 4, curve (*c*). The microscopical condition of the fibres has also been altered, though not so much as with other methods of drying. The appearance of these skins after soaking in water, hydrochloric acid and sodium hydroxide respectively is described in Table VI. Treatment of the skins with trypsin restores the swelling power (curves (*b*) and (*d*), Fig. 4), a fact which suggests that the drying affects not the collagen fibres themselves, but rather the accessory structures in the skin.

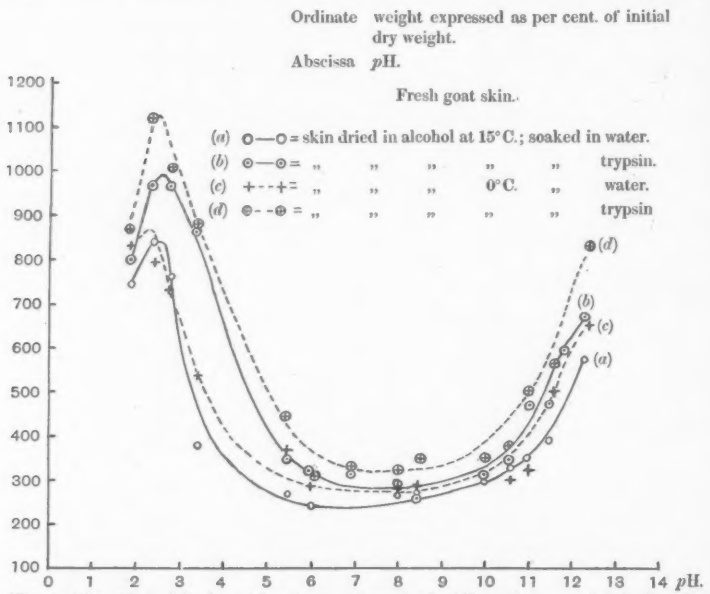

Fig. 4. The effect of drying at low temperatures and of dispersing coagulated albumin in skins so dried.

Comparative experiments were made with skin dehydrated with alcohol at 15°. The quantitative differences between drying at 0° and 15° are not great (Fig. 4, curves (*a*) and (*c*)), but the qualitative differences are marked, as shown in Table VI, and particularly in Table IV.

Up to the present no conditions have been found under which a skin once dried can subsequently be restored exactly to the fresh condition. Even under conditions chosen to produce the least chemical disturbance of the skin proteins (alcohol at 0°) it can be seen (Table VI (*a*)) that the freedom of the fibrils has largely been lost, with the accompanying reduction of the power

Table VI. *Fresh goat skin dried out in alcohol at 0° and 15°. Swelling in acid, water and alkali with and without treatment with trypsin. Microscopical examination.*

p_H at equilibrium	Dried in alcohol at 0°		Dried in alcohol at 15°		Fresh goat. Undried. Control
	Water soaked (*a*)	Trypsin soaked (*b*)	Water soaked (*c*)	Trypsin soaked (*d*)	
2·4	All fibres swollen. Split well at the ends into fibrils and shew fibril striation down their lengths	Fibres swollen. Well split at ends and along the lengths of the fibres into fibrils	Fibres not swollen as much as (*a*) or (*b*) but show signs of splitting along the fibres and also at the ends	Fibres swollen, partially split along the lengths and well split at the ends	Fibres fully split into fibrils and well swollen. Encircling bands numerous
8	Fibres not swollen; not confined into bundles and are not split into fibrils but show striations. On swelling with acid the fibres split more into fibrils, but show no encircling bands	Fibres well split into fibrils; these are well separated—much more than in the water-soaked skin. Fibrils not swollen. Swell and split further in acid solution but show no encircling bands	Fibres split into fibrils in many places. All bundles are ruptured. Fibrils not swollen; swell and split more in acid solution, but do not swell to the same extent as (*a*) and (*b*)	Fibres well split into fibrils. All bundles are ruptured. Fibrils not swollen. Swell and split more in acid solution, but do not swell to the same extent as in (*a*) and (*b*), and show no encircling bands	Fibres thin, unswollen and wavy; confined into bundles; individual fibrils clearly visible. On swelling with acid the encircling bands appear.
11·8	Fibres swollen but not so much as in acid. Fairly well split into fibrils but these are not separated	Fibres swollen but not so much as in acid. Split and separated into fibrils	Fibres separated and swollen but not as much as in acid. Split and well separated into fibrils	Fibres swollen but not so much as in acid. Split and well separated into fibrils	Fibres split into fibrils and swollen but not as much as in acid. Some encircling bands present

All fibres, when teased out of the skin, swell more in the equilibrium solutions. This extra swelling is greater in acids than in alkalies. It does not, of course, occur in water.

The waviness of the fresh fibril has been lost in all the dried fibrils, and the encircling bands characteristic of the swollen fresh fibre-bundles have also been destroyed.

of colloidal swelling (Fig. 4, curve (*c*)). Both the loss of freedom of the fibrils and also the swelling power can be restored by the action of trypsin (Table VI (*b*) and Fig. 4, curve (*d*)), but the action of the enzymes must have been to remove some of the constituents of the fresh skin. Other changes caused by drying, such as the loss of wavy outline of the fresh fibril, and the rupturing of the encircling bands of the fibre bundles, are also permanent.

II. The Chemical Basis of Skin-Swelling.

Microscopical investigations described in a previous paper [1924] show that the swelling of skin in strong acids or alkalies, is due entirely to the colloidal swelling of the collagen fibrils. Neither the keratins of the epidermis nor the elastin of the elastin fibre network becomes swollen under conditions which cause marked swelling of the collagen fibres. The absorption of water by the latter is a process parallel in many ways to its absorption by gelatin, but the fibrous structure present in skin has an important influence on the quantity absorbed.

Both gelatin and skin, when swelling in an acid or an alkaline liquid, absorb not only water but also either acid or alkali, as the case may be. An investigation has been made on the quantity of acid (or alkali) absorbed by skin when the fibrils are in various degrees of mechanical freedom, and the results have been compared with results previously obtained for gelatin. Fig. 5 shows the curve of absorption of hydrochloric acid by goat skin. The number of equivalents absorbed by 10 *g.* of dry skin is taken as ordinate and the reaction of the external solution as abscissa. Experimental points are shown for the absorption of acid by (i) fresh goat skin, (ii) sun-dried goat skin, (iii) sun-dried skin which had previously been treated with ammonia, or (iv) with salt solution. It has previously been shown that the water absorption of skin in these four different conditions varies considerably. Their absorption of hydrochloric acid is the same within the limit of experimental error, i.e. the mechanical freedom of the fibrils does not affect their relations towards the hydrochloric acid molecule. The curve for absorption of hydrochloric acid by gelatin (plotted from values given by Procter [1914]) is also given in Fig. 5. The resemblance between the two curves suggests that the chemical basis of the absorption of the acid is the same in the two cases. The acid absorbed is not all *chemically* combined; part is present as free acid. Fig. 5 also gives the curve of combined acid for gelatin, the values being again taken from Procter's paper. The curves of *absorbed* and *combined* acid are coincident on the scale of ordinate used in Fig. 5 up to a reaction of about p_H 2·7. After that they become increasingly divergent. Absorbed acid in a distended skin must therefore not be assumed to be all combined, except at acid reactions below p_H 2·5, where the establishment of a membrane equilibrium would result in the expulsion of most of the free acid from the system. At more acid reactions the membrane equilibrium would persist, but increasing external concentration would drive acid into the system.

The absorption of alkalies by skins follows a similar course to the absorption of acid, but in the absence of the corresponding data for gelatin, a detailed analysis of the experimental values has not been made. The absorption of alkali is not affected by the structural condition of the skin.

The actual quantity of acid (or base) combined with the skin proteins at any reaction cannot be calculated from any data at present available. In view of the mixed composition of the skin any definite value, even if obtained, would have little meaning.

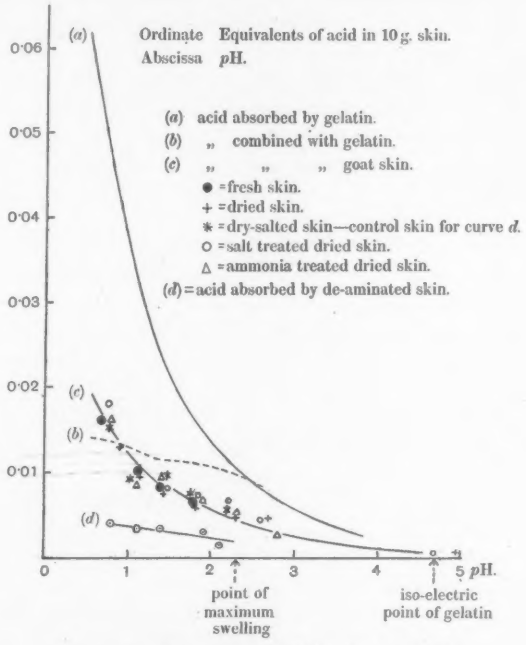

Fig. 5. The absorption of acid by gelatin and skin.

The swelling of gelatin gels which occurs both in acid and in alkaline solutions in excess of that which would occur in water only, can be attributed to the osmotic pressure of the ionised salts formed by the gelatin with the acid or alkali. Procter [1914] first explored the mathematical basis of this theory, and gave equations which are in good agreement with experimental facts. On this theory the free-NH_2 groups of the gelatin molecule serve as the points of attachment for the acid (see Jordan Lloyd, 1920, and Jordan Lloyd and Mayes, 1922).

If the swelling of gelatin in acid solutions is due to salt formation by addition at the $-NH_2$ groups, a crucial test would be to remove these and use the de-aminated gelatin as experimental material. A quantity of gelatin

was de-aminated by the method of Blasel and Matula [1914]. After dialysis for three weeks against distilled water the precipitate was dissolved in a small volume of warm water, re-precipitated with alcohol, dried with alcohol and ether and stored in a vacuum desiccator. This de-aminated gelatin in powder form was submitted to the full range of acid and alkaline reaction. The volume occupied by unit weight of the powder was measured and compared with that occupied by unit weight of gelatin powder of the same average diameter. De-aminated gelatin is very soluble in dilute alkalies, but in all the alkaline solutions the volume occupied by both the de-aminated and the control powders was the same within the limit of the experimental error. This shows that de-amination has not affected swelling in alkali. In the acid solutions, the swelling of the de-aminated gelatin was about half that of the control. The measurement of swelling by the volume changes of powders is not very accurate and all that can be safely deduced from this experiment is that de-amination reduces the power of swelling in an acid solution.

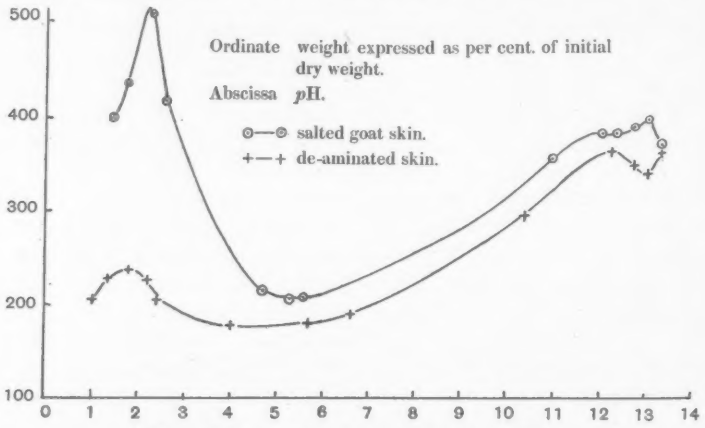

Fig. 6. The effect of de-amination on skin swelling.

It has been shown that the curves of increase of weight plotted against reaction are closely parallel in gelatin and in skin. A piece of dried goat skin[1] was therefore treated by the same method used to prepare de-aminated gelatin. It was dried and the swelling curve was taken for both acid and alkaline solutions. This is shown graphically in Fig. 6, together with the curve for the untreated control. It will be seen that in the de-aminated skin there is very little swelling in acid solutions. This is not due to any alteration in the physical state of the skin fibres, since the two curves of alkaline swelling lie very close together. It is impossible at the moment to say whether the slight swelling that occurs between p_H 3 and p_H 1 is due to incomplete de-amination or whether it is due to an entirely separate swelling mechanism. Separate fibres were

[1] This material was kindly prepared for us by Dr E. W. Merry.

teased out from the edge of the skin and the change in volume examined under the microscope, using distilled water, sodium hydroxide solutions (0·05 *N* and 0·02 *N*) and hydrochloric acid solutions (0·01 *N* and 0·002 *N*). The results are given in Table VII, together with figures for dried goat fibres as controls. It can be seen that in the alkaline solutions the swelling of both kinds of fibre is of a similar order, whereas in acid solutions the swelling of the de-aminated fibres is not markedly in excess of that obtained in the distilled water.

Table VII. *Swelling figures for fibres.*

	(A) From de-aminated dried goat skin			(B) From dried goat skin		
Solution	Change in length from 100 to	Change in breadth from 100 to	Change in volume from 100 to	Change in length from 100 to	Change in breadth from 100 to	Change in volume from 100 to
Distilled water	102	138	194	116	115	153
NaOH 0·05 *N*	109	211	485	90	182	298
NaOH 0·02 *N*	97	201	392	75	246	454
HCl 0·01 *N*	101	162	265	70	298	624
HCl 0·002 *N*	104	140	204	67	416	1160

The curve of absorption of hydrochloric acid by de-aminated skin is given in Fig. 5, curve (*d*). It lies considerably nearer to the horizontal axis than that of the unde-aminated control (*c*). This fact proves that some of the acid is combined at those free amino-groups which can be removed by nitrous acid. The curve of absorption of alkali is the same in the de-aminated material as the control, within the experimental error of the method of measurement. Recently Hitchcock (1923) has shown that de-amination by nitrous acid reduces the power of gelatin to combine with acid by about one-half. This corresponds closely with the results obtained for skin, which are summarised in curves (*c*) and (*d*) in Fig. 5. At p_H 2 the absorption of acid by the skin is reduced by de-amination to about a half. At more acid reactions the reduction is still greater, but if the curves (*a*) and (*b*) for acid absorbed and combined by gelatin are compared with (*c*) the curve of acid absorbed by skin, and (*d*) the curve of acid absorbed by de-aminated skin, it seems most likely that the rise in curve (*c*) at reactions more acid than p_H 2 is due to acid which is absorbed only but not combined. Curve (*d*) does not show this later rapid rise, and therefore the relations of the two curves at or near p_H 2 would seem to indicate the true relations of the combined acid in the two systems. At this reaction, judging from curves (*a*) and (*b*) the greater part of the absorbed acid is combined with the protein, and the value for the de-aminated skin is about half that for the unde-aminated.

The experiments given above show that the absorption of water, acid and alkali follows similar lines in a system such as a gelatin gel and a tissue of collagen fibres such as skin. The colloidal swelling in both cases is due to the combination of an ampholyte with either acid or base, with the production of ionisable salts which draw water into the system by an osmotic force. With

both gelatin and collagen, combination with acid, and acid swelling are shown to depend largely (but not entirely) on the presence of free amino-groups which can be removed by means of nitrous acid. The combination of the protein with the acid (or base) is independent of the histological structure, if any, of the system, i.e. the chemical equilibrium is independent of histological structure within the error of the experiment. On the other hand, the absorption of water as the result of osmotically active protein salts is undoubtedly largely influenced by the mechanical condition of the system.

We should like to take this opportunity of thanking the Director and Council of the British Leather Manufacturers' Research Association for permission to publish this work.

Summary.

1. The fibrils and fibres of skin are embedded in a matrix, the interfibrillary fluid, the properties of which closely resemble those of the plasma proteins.

2. In skins which have been allowed to dry under conditions which lead to the coagulation of albumin, such as at a temperature above blood heat, in a strong light, in the presence of alcohol at room temperature, the interfibrillary proteins form a non-swelling, insoluble, non-elastic deposit round the fibres and the water absorbing power of the skins is reduced. Such skins do not soften when soaked in water.

Substances which disperse or destroy denaturated albumin, such as sodium citrate or trypsin, remove this coagulated interfibrillary matter, and skins so treated have a greater capacity for water absorption, though this is not restored to the level found in fresh skins.

3. A fresh skin has a greater capacity for absorbing water than a skin which has been dried even when dehydration has been carried out by means of alchohol at 0°.

The water absorbing power of skins which have been dried at 37° or less, can be brought near to the level of that of fresh skins by treatment with trypsin. The drying therefore has not affected the fibrils themselves so much as the interfibrillary proteins.

4. The colloidal swelling of skins is due to the network of skin-fibres. These contain all the collagen found in the skin. The swelling of skin in acid and in alkaline solutions is due to the formation of ionisable salts of collagen.

The absorption of acid or alkali by skins is not influenced by the structure.

5. Swelling in acid solutions is shown to be due to the presence of free amino-groups in the skin. Removal of these groups reduces the amount of acid both absorbed and combined. Near the reaction of maximum swelling, where the absorbed acid is almost all combined, the reduction in the amount of acid absorbed brought about by de-amination is about one-half.

REFERENCES.

Blasel and Matula (1914). *Biochem. Z.* **58**, 417.
Chick and Martin (1910). *J. Physiol.* **40**, 404.
—— —— (1911). *J. Physiol.* **43**, 1.
—— —— (1912, 1). *J. Physiol.* **45**, 61.
—— —— (1912, 2). *J. Physiol.* **45**, 261.
Eitner (1880). *Der Gerber*, 112.
Hardy and Gardiner (1910). *J. Physiol.* **40**, *Proc.* lxviii.
Harris (1923). *Proc. Roy. Soc. London*, A, **94**, 246.
Hartley, Eagleton and Okell (1923). *J. Path. Bact.* **26**, 53.
Hitchcock (1923). *J. Gen. Physiol.* **6**, 95.
Kaye and Jordan Lloyd (1924). *Proc. Roy. Soc. London*, B, **96**, 293.
Jordan Lloyd (1920). *Biochem. J.* **14**, 147.
Jordan Lloyd and Mayes (1922). *Proc. Roy. Soc. London*, B, **93**, 69.
Mclaughlin and Theis (1923). *J. Amer. Chem. Ass.* **18**, 324.
Michaelis and Rona (1910). *Biochem. Z.* **27**, 38.
Procter (1914). *J. Chem. Soc.* **105**, 313.
Rosenthal (1916). *J. Amer. Leather Chem. Ass.* **11**, 463.
Young (1922). *Proc. Roy. Soc. London*, B, **93**, 235.

CXXXVII. A CRITICAL STUDY OF THE METHODS OF ESTIMATING CHOLESTEROL AND ITS ESTERS IN TISSUES, PART II.

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The methods in use for the estimation of cholesterol and its esters in tissues are liable to many quite distinct sources of error. Errors may arise through faulty sampling, unsuitable methods of preparing the tissue for extraction, inadequate extraction, incomplete hydrolysis of esters, and lastly through defects inherent in the methods used for estimating the cholesterol in the extracts. The errors to which the gravimetric and colorimetric methods of estimating sterols are liable have been already fully discussed in Part I by Gardner and Williams [1921] and by Gardner and Fox [1921].

In this paper we propose to deal with the other sources of error and more particularly that due to incomplete hydrolysis of the esters.

Sampling.

It is not safe to cut off a piece of a weighed organ for analysis and assume that the percentage of sterol found gives a representative value for the whole. The whole organ should be finely minced and thoroughly pulped in a mortar and an aliquot portion taken. Even this is not safe in the case of tissues containing much intercellular fat. For example, two aliquot portions of a very carefully pulped mixture of several suprarenals gave 3·46 % of ether extract and 0·334 % of cholesterol in one and 3·40 % and 0·305 % in the other.

If the necessary apparatus is available greater uniformity of the material can be obtained by freezing the pulp and grinding the frozen mass. The most satisfactory method, however, is to extract, when possible, the whole of the pulped organs, and make the extract up to known volume and then take an aliquot portion for analysis. This is, however, rather troublesome in the case of large organs such as liver.

Preparation of the material for extraction.

If the material is to be directly extracted by ether or by any solvent immiscible with water, it must be dried. Unsuitable methods of drying are

fruitful sources of error. Drying by heat, particularly at 100°, is quite unsuitable. It has long been recognised that material dried in this way yields only a portion of its ether-soluble constituents to direct extraction by ether, though alcohol is much more effective. Even when a complete extraction has been effected, the ether extract finally obtained is always deep brown in colour; and in the estimation of the free cholesterol by digitonin the colouring matter is sometimes carried down with the cholesterol digitonide precipitate and is difficult to get rid of without danger of dissolving some of the digitonide itself.

The drying of the ether extract itself by too prolonged heating also appears to modify the ratio of free to bound cholesterol.

Thaysen [1914] recommends the drying technique used by Rübow [1904] and by Erlandsen [1907] in which the pulped tissue is dried in thin layers in a current of air at room temperature or at 28°–32°. After drying for 10 hours the material is finely powdered in a mill and the drying finished *in vacuo* over sulphuric acid for 48 hours. This process is very tedious, but we have found it gives as a rule a colourless or pale yellow extract. Other workers have dried the tissue pulp by mixing with anhydrous sulphate of soda [Fränckel and Elfer, 1910] or with plaster of Paris [Windaus, 1910; Rosenheim, 1906; Gardner and various co-workers]. When the mixture has set to a solid mass it is powdered and extracted. The ether extract obtained in this way is but slightly coloured.

Extraction of the ether-soluble constituents.

Thaysen [1914] has investigated the successive extraction of various tissues and fluids, dried by his method, by ether, alcohol-ether, and hydrolysis by soda and extraction by ether. The powdered dry organ was first extracted with ether in a Soxhlet for 48 hours, then exhausted with alcohol in a beaker at 60°–70° for 5–6 hours and again extracted with ether in a Soxhlet. The alcohol extract was worked up with the second ether extract. Finally the extracted powder was placed in a flask with 25 cc. of 20 % aqueous caustic soda and heated in a water-bath for 3–4 hours; then it was poured into a dish, neutralised with hydrochloric acid and evaporated to dryness. The residue was then extracted with ether. In the three ether extracts thus obtained the quantities of free and bound cholesterol were estimated by Windaus' digitonin method.

It is clear from his results, making ample allowance for errors of experiment in the estimation of fractions of milligrams, that both simple ether and alcohol-ether extraction give very imperfect results for air-dried serum. In the case of serum dried by sulphate of soda he obtained better results by ether extraction but they were still too low. He concludes from his investigations that cholesterol and cholesterol esters are bound in the serum in another manner than in the organs, and that in the "serum" itself they circulate in a more or less loose combination with other material. It has been suggested, though without much real proof, that the proteins combine with the sterols.

Bang, for instance [1911], states that the cholesterol esters in a peritoneal fluid are chemically bound to euglobulin.

Thaysen generally concludes that alcohol-ether extraction is a good reliable method; but even simple ether extraction gives very useable results in the case of tissues.

It can be seen from Thaysen's results that simple 48 hours' ether extraction takes out in the case of two samples of liver 99·07 and 97·3 % of the total sterol: in kidney 99·6 and 98·2 %: in suprarenals 99·16 %: and in heart muscle 98·2 and 95·2 % of the total.

In their earlier papers Gardner and Dorée, Ellis and Lander relied on getting the whole of the sterols from tissue dried with calcium sulphate by prolonged ether extraction, usually 10–14 days. This assumption was probably justified, since their values for various tissues are of much the same order as those obtained by Thaysen himself and by later workers by other methods. Lapworth also [1911] showed that drying with calcium sulphate or sulphate of soda did not lead to any appreciable esterification of the free cholesterol. J. Fex [1920] conclusively showed that ether extraction of liver tissue, dried with sulphate of soda, gave quite correct results.

To get over the difficulty of imperfect extraction of dried serum or dried tissue two methods have been used by various observers: (1) use of several solvents, (2) destruction of protein matter by alkalis prior to extraction with ether. Kumagawa and Suto [1908] worked out the relative efficiency of various solvents in extracting fat from heat dried tissues.

Rosenfeld [1900] introduced a method for extracting fatty matter from tissues, which has been much used, and which he claimed gave good results. The tissue is dried with alcohol and heated on a water-bath in a beaker covered by a watch-glass and containing alcohol. This appears to break up any combination, chemical or physical, of the fat with protein. After pouring off the alcohol the residue is extracted in a Soxhlet with chloroform for 4 to 6 hours. The treatment with alcohol and subsequent extraction with chloroform is again repeated. The extracts got by evaporating the alcohol and chloroform solutions separately to dryness are taken up in ether, filtered and the ether evaporated.

The writers have obtained satisfactory extraction by drying the tissue in alcohol, then extracting in a Soxhlet first with alcohol for 24 hours or more and finally with ether. Care must be taken to avoid rubber or cork stoppers. The extracts are evaporated to dryness and taken up in either ether or light petroleum. In our experience a perfect extraction is attained by this method, but the ether extract finally obtained is apt to be dark in colour and sometimes yields a coloured digitonin precipitate in the subsequent sterol estimation.

Other workers destroy the protein by heating with alkali before extracting the sterols. In 1908 Kumagawa and Suto worked out their well-known method for the estimation of fat and unsaponifiable matter in tissues and blood. The method was scarcely new, as a similar procedure had been previously proposed by Liebermann and Székely [1898].

The minced organ is boiled 2 hours with 20 % caustic soda. The solution is placed in a separator, cooled and 20 % hydrochloric acid added in excess, whereby a copious precipitation takes place. Plenty of ether is now poured in and the whole well shaken. After this has stood for some time the ether separates from the other fluid and the precipitate forms a thin layer under the ether. The undermost clear fluid is drawn off, and the upper ether poured off. The precipitate left in the separator is then dissolved in caustic soda and the alkaline solution shaken out with a fresh portion of ether several times. The various ether extracts are combined, evaporated and the residue dried. It is then dissolved in light petroleum. This solution contains the liberated fatty acids and unsaponifiable matter. To get rid of the fatty acids the petroleum solution is brought into a separator and absolute alcohol containing 1 % of potash is added. Water is now added and on standing two layers separate, the soaps in the alcohol layer and the sterols in the light petroleum layer. The advantages of the method are that we get a quantitative extraction, *provided the extraction with ether is repeated a sufficient number of times*. The total cholesterol is in a form suitable for digitonin estimation, and *fresh* tissue or serum can be used without drying.

Since this method was published a large number of methods have been described for estimating cholesterol by colour reactions. These methods have mostly been applied to blood serum. The methods are generally similar, mostly differing in details of extraction, and give fairly similar results. The errors in colorimetric methods have been fully dealt with in Part I, and have also been discussed by Fex [1920]. We only concern ourselves here with the extraction.

Grigaut [1913] mixes 20 cc. serum with 20 cc. caustic soda of 36° Be (i.e. 400 g. per litre) and heats in an autoclave at 110° for one hour. In the case of tissues 5 to 10 g. of the fresh tissue with 40 cc. alkali diluted by half to correspond with the serum conditions are treated similarly. The fluid obtained is shaken in a separator with 60 cc. of ether at 40°–45°. Six successive shakings suffice. When the layers have separated, the lower is drawn into a flask and after heating to 40°–45° is again extracted. The ether is then evaporated and leaves a residue containing cholesterol and some impurities. In order to remove these the residue is dissolved in 50 cc. of 95 % alcohol with addition of 1 cc. of alcoholic soda (1 in 100). He then evaporates to dryness on a water-bath and takes up with light petroleum. On standing, impurities subside and can be easily filtered. The petroleum solution is allowed to evaporate spontaneously and the cholesterol separates in needles and can be weighed after drying at 100°. According to Grigaut, this gives the total cholesterol free and ester. In his colorimetric method 2 cc. of serum are placed in a special cholesterimeter, then 13 cc. of alcohol of 60° containing 1 in 200 of caustic soda. After mixing he adds 15 cc. ether, stoppers the instrument and shakes. After standing he runs off the aqueous layer and replaces by 20 cc. of water. After shaking and standing 5 minutes he again runs off the water and washes with another 20 cc. After removing the water the ether is evaporated in a

porcelain dish. The residue is taken up in 5 cc. of chloroform for the colour test. According to Grigaut, the alcohol by its simple presence is capable of assuring the total passage of the cholesterol into the ether.

Autenrieth and Funk [1913] digest the measured serum on a water-bath for 2 hours with 10 volumes of 25 % caustic potash. The fluid is then extracted with twice its volume of chloroform by shaking for 5 minutes. The chloroform is then separated and the extraction repeated four times. The chloroform extracts are finally dried with sulphate of soda.

Weston and Kent [1917] mix serum with 10 volumes of 95 % alcohol and warm at 60° for 24 hours. The alcohol is filtered and the residue boiled with alcohol. It is then digested 24 hours with ether. The combined extracts are boiled with a few grams of caustic potash for 2 hours, saturated calcium chloride solution added and the precipitate collected on a filter. This is washed with lime-water and extracted with chloroform.

Czonka [1916] takes 2 cc. serum, 20 cc. alcohol and caustic potash and 2 cc. 10 % barium chloride. This mixture is boiled under a reflux for one hour, and after cooling extracted with ether. The ethereal extract is washed with water, evaporated, and the residue taken up in chloroform.

Fex [1920] digests fresh tissue, or blood or serum with excess of 2 % caustic soda solution until the mass becomes gelatinous and goes partly into solution. He then heats on a water-bath for $\frac{1}{4}$ to $\frac{1}{2}$ hour until nearly everything dissolves. The alkaline fluid is then repeatedly extracted with ether.

Bloor [1916] pours the blood or serum in a thin stream into a mixture of three parts of alcohol to one of ether, gently shaken. The mixture is then placed in warm water until the ether begins to boil. It is then cooled and made up to known volume with the alcohol ether mixture.

In processes involving the use of alkali more or less hydrolysis of sterol esters by the alkali takes place, the amount depending on the strength of alkali, temperature and time.

In the colorimetric methods it is assumed that cholesterol and cholesterol esters give the colour reactions with acetic anhydride and sulphuric acid in the same manner, so that it is of no importance whether any hydrolysis is brought about by the alkali or not; the result is the total cholesterol free and combined. That this assumption is not justified, has been pointed out by Gardner and Williams [1921] who showed that in the Liebermann reaction the colour-changes develop at different rates in the case of free sterols and esters, and that in either case variation in the conditions under which the reaction develops has a marked effect on the result.

It is well known that both the gravimetric digitonin method and the colour methods based on Liebermann's reaction (when properly done) give accurate results when pure cholesterol is used. When applied, however, to tissue extracts the two processes often give discordant results. The digitonin process gives very accurately the free cholesterol, and the cholesterol liberated by hydrolysis of esters, *but in the latter case the value of the figure is conditioned by*

the efficiency of the hydrolytic process used. Thaysen, from experiments on cholesterol oleate, maintains that the esters should be boiled for at least 8 hours, and preferably 12, with an alcoholic solution of sodium ethoxide. He used an ester solution in alcohol of about 1·8 %, but did not make it clear what quantity of sodium he used.

We have been in the habit of estimating total cholesterol in an extract by dissolving in ether and adding 10–20 times the theoretical quantity of sodium *ethoxide* in alcohol. As a rule under these conditions hydrolysis is complete on standing overnight in the cold. This procedure has the advantage that less coloured resinous matter is produced than by boiling with alcoholic sodium ethoxide. The ether washings from the cholesterol digitonide should, however, always be tested with Liebermann's reagent to make sure that the hydrolysis was complete. Mueller [1916] has shown that on the same serum Bloor's method gives higher results than that of Autenrieth and Funk, and the latter higher values than the digitonin method. Fex [1920] made an extensive series of estimations of known cholesterol and cholesterol ester solutions by Autenreith and Funk's method and got results varying in rather wide limits about the mean: he also discussed at length the Grigaut, Bloor and other methods and came to the conclusion that if we wish to attain a certain knowledge of the actual absolute quantities of cholesterol, free and combined, which are contained in blood and tissues, these methods cannot be used. We consider, however, his conclusion too sweeping. We think that there can be no doubt, from a careful survey of different workers' results, that errors due not only to deficient extraction, but to incomplete hydrolysis of esters account very largely for the many conflicting results.

Hydrolysis of cholesterol esters.

It seemed to us, therefore, that an investigation of the hydrolysis of esters, likely to occur in animal tissues and fluids under various conditions, would enable us to form some estimate of the errors that might arise through inadequate hydrolysis, and possibly to get some criterion of the relative values of the data accumulated by different observers.

For this purpose we selected the three esters which commonly occur and form the bulk of the sterol esters of animal tissues—cholesterol oleate, palmitate and stearate. We also investigated cholesteryl arachidate, which is stated to occur in tissue, and cholesteryl cerotate as an example of an ester difficult to hydrolyse. The esters were in every case tested with digitonin to prove the absence of any free cholesterol. Hydrolysis was investigated under the following conditions: (1) the ester was soaked overnight in 2 % caustic soda solution, and then heated for one hour on a water-bath, as in the method of Fex for treating tissues; (2) heated for 4 hours with 25 % aqueous caustic soda sometimes on the water-bath, but generally in a bath of sulphuric acid so that the fluid actually boiled and kept the difficultly soluble esters well mixed with the alkali; (3) boiled for 4 hours with *N* alcoholic soda; (4) the ester dissolved in

ether, saturated alcoholic sodium ethoxide added and allowed to stand in the cold for 24 hours, conditions used by us in the hydrolysis of tissue extracts.

A very large excess of alkali was used and the quantity of alkali was kept approximately the same per gram of ester. Further the quantities taken for analysis were of the order that would be dealt with in ordinary analyses of tissues. In the case of (1) and (2) the alkaline fluid, diluted if necessary, was thoroughly extracted with ether. In (3) the alcoholic solution after hydrolysis was largely diluted with water and thoroughly extracted with ether. The ethereal solution was then well washed with water. In (4) the hydrolysate was diluted with ether and shaken repeatedly with water to get rid of excess of alkali, soap, alcohol, etc. The wash waters were collected and shaken with ether. The combined ethereal solutions were then again washed with water, evaporated to dryness, taken up in alcohol and precipitated with excess of digitonin. The estimations were made according to the procedure of Fraser and Gardner [1910]. The results are given in the Table, p. 1065.

Cholesteryl oleate was prepared by heating cholesterol with excess of oleic acid to 200°, according to the method of Hürthle [1895]. It melted at 41°–42°, and had a specific rotary power $[\alpha]_D$ − 18·5°. A test with digitonin proved it quite free from uncombined cholesterol.

Cholesteryl palmitate was prepared by the method of Abderhalden and Kautzsch [1910] by the action of palmityl chloride (Kahlbaum) in chloroform solution on dry cholesterol. It melted at 77°–78° and had a specific rotation in chloroform $[\alpha]_D$ − 24 ·2°. Tests showed it to be quite free from uncombined cholesterol.

Cholesterol stearate was obtained in good yield by heating 12 g. cholesterol in chloroform solution with 14 g. stearyl chloride (Kahlbaum). It was purified by repeated crystallisation from alcohol. Abderhalden and Kautzsch, who first prepared it in this manner, gave the melting point as 85°–90°. They gave no rotation and their combustion figures suggested that their preparation was not pure. Our preparation melted sharply at 81°–81·5° and had a specific rotation in chloroform $[\alpha]_D$ − 27·6°. A test showed it free from uncombined cholesterol. It seemed to be somewhat more readily hydrolysable than the palmitate.

Cholesteryl arachidate. We have found no previous record of the preparation of this ester, which we carried out by the method of Abderhalden and Kautzsch. 12 g. of arachidic acid were converted into arachidyl chloride by boiling with 25 g. thionyl chloride. The crude arachidyl chloride was dissolved in chloroform and boiled with the theoretical quantity of dry cholesterol. The ester separated on dilution with methyl alcohol. It was purified by recrystallisation from boiling alcohol, from which it separated in white flakes which bound together on rubbing. It melted at 76°–79·5° and in chloroform solution gave a specific rotation $[\alpha]_D$ − 22·2°. 100 cc. ethyl alcohol at the boiling point dissolved 0·335 g. and 100 cc. of cold ether 0·856 g. A digitonin test showed it free from uncombined cholesterol, and an estimation of the cholesterol after hydrolysis showed that it was pure.

Cholesteryl cerotate. We had some difficulty in making this compound as we were unable to purchase pure cerotic acid. According to Marie [1896], cerotic and melissic acids occur together as esters in carnauba wax and Chinese wax and are not easy to separate and purify. Two specimens of commercial acid melted at 66°–67° and 63°–65° respectively. We were unable to ascertain the source from which these specimens were made, but both contained unhydrolysed wax. The crude acid was boiled with an alcoholic solution of sodium ethoxide. The alkaline soap obtained was thoroughly washed with ether and the soap solution extracted with ether. From the ethereal extract we obtained an alcohol melting at 82°–83°, perhaps myricyl alcohol. The acid liberated from the soap by mineral acid was taken up in ether. After evaporation of the ether, the acid was repeatedly recrystallised from alcohol, in which it was very difficultly soluble. The final crop melted at 80°–82° and separated from the alcohol as a white powder. The melting point of cerotic acid is given in the literature as 77·5° and of melissic acid as 90°–90·6°. Melissic is less soluble in methyl alcohol than cerotic acid.

Our acid was probably mainly cerotic with some melissic acid. For our purpose this did not much matter, since our object was to ascertain whether esters of such acids were more difficultly hydrolysable than those of palmitic, stearic, etc. 6·8 g. cerotic acid were converted into chloride by means of thionyl chloride, and the crude cerotyl chloride taken up in chloroform and boiled with 7 g. dry cholesterol. The ester separated on pouring into alcohol. It was purified by repeated recrystallisation from a large volume of boiling alcohol in which it was only sparingly soluble. It separated as a white crystalline powder, which on heating began to soften at 65° and melted at 73°–75°. In chloroform solution it had a specific rotation $[\alpha]_D$ − 5·3° approximately. 100 cc. of boiling ethyl alcohol dissolved 0·296 g. A test with digitonin showed that it contained a minute trace of cholesterol—0·0004 g. in 0·1857 g., i.e. about 0·2 %.

The results of the hydrolysis of these esters under the conditions mentioned above are summarised in the following Table:

		Heated 4 hours with 10 cc. of 25 % aqueous caustic soda			Boiled 4 hours with 60 c.c. of *N* alcoholic soda			Ether solution of ester allowed to stand 24 hrs. in cold with alcoholic sodium ethoxide		
Ester	2 % caustic soda solution	wt. of ester, g.	wt. of digitonin comp., g.	% hydrolysis	wt. of ester, g.	wt. of chol. digitonide, g.	% hydrolysis	wt. of ester, g.	wt. of digitonin comp., g.	% hydrolysis
Oleate[1]	No action	0·0516	0·1283	100	0·0493	0·1340*	100	0·0504	0·1266*	100
Palmitate	,,	0·0582 0·0510	0·0582 0·0301	39 23	0·0621	0·1633	100	0·0488	1·1364	100
Stearate	,,	0·0481	0·1124	95–96	0·0520	0·1353*	100	0·0497	0·1180	98
Arachidate	,,	0·0438 0·0514	0·0915 0·1060	89 88–89	0·0521	0·1248	100	0·0391	0·091	100
Cerotate[2]	,,	0·0371	0·0346	45	0·0566	0·0433	65	0·1147	0·2004	84

* These digitonides were slightly coloured by resinous matter.

[1] In an experiment in which cholesteryl oleate was allowed to stand 24 hours in the cold with alcoholic sodium ethoxide, without the addition of ether, hydrolysis was incomplete; 0·0368 g. of oleate gave only 0·0612 g. of compound, corresponding to 68 % hydrolysis. This was no doubt due to the sparing solubility of the ester in cold alcohol, for even at the boiling point 100 cc. of alcohol only dissolve 0·4 g. of the ester.

[2] The cerotate when refluxed in ether solution with sodium ethoxide underwent 86 % of hydrolysis and was completely hydrolysed by 4 hours' boiling with alcoholic sodium ethoxide.

We consider that these experiments afford an experimental justification of the method adopted by one of us and various co-workers for the hydrolysis of esters in tissue analysis, viz. treatment of an ethereal solution with excess of alcoholic sodium ethoxide in the cold.

They also afford some explanation of the varying results obtained in comparative experiments by the methods of Bloor, Autenrieth and Funk, and Grigaut, since, as has been shown by Gardner and Williams [1921], the cholesterol esters develop colour with the Liebermann reagent at quite different rates and in some cases with different tints from pure cholesterol. The colour reaction methods when applied to tissues are also rendered less precise owing to the substances of non-sterol character which accompany cholesterol and its esters in the tissue extracts and which give more or less brown coloration with acetic anhydride and sulphuric acid and modify the green tint due to the cholesterol so that comparison with the pure green tint of the standard is difficult. This is also accentuated as we have previously shown by the production of traces of resinous bodies during hydrolysis which also give yellow-brown or greenish colours with the Liebermann reagent. The colour methods also have the disadvantage of being micro methods, so that errors of experiment inherent in the most skilful work become enormously magnified when results are calculated to percentages. These hydrolysis experiments also threw light on some of the results obtained by one of us [Gardner, 1921] on the sterols of faeces. In that paper it was shown that after removing free sterols by digitonin, hydrolysing the esters by means of the action of a large excess of sodium ethoxide in an ethereal solution of the material and again removing the sterols, thus set free, by means of digitonin, a residual oil could be obtained which was capable of being distilled in superheated steam or in a high vacuum without decomposition. The purified oil thus obtained gave as a rule a brown colour in chloroform solution with acetic anhydride and sulphuric acid, but in some cases the brown gradually changed to a dusky green. The latter oils on hydrolysis in sealed tubes with alcoholic caustic soda in large excess yielded traces of sterols again precipitable by digitonin. Evidently the faecal waxes contained small quantities of stable esters such as cholesterol cerotate, which escaped hydrolysis by sodium ethoxide in ethereal solution. Possibly such esters may occur also in minute quantity in tissues and body fluids, and in order to insure complete hydrolysis of an ether extract it is perhaps advisable to boil in alcoholic solution with sodium ethoxide for several hours.

Since the cholesterol esters that occur in tissues are quite unaffected by a 2 % solution of caustic soda, Fex's method of extraction presents distinct advantages. His procedure is as follows. The required weighed quantity of minced fresh tissue is mixed with about twice its weight or more of 2 % caustic soda. It is allowed to digest at room temperature for several hours until the tissue swells up and a gelatinous transparent mass is obtained. The flask is then put on a boiling water-bath until the gelatinous stuff goes largely into solution. This generally takes about half an hour. A brownish-red

moderately clear liquid is obtained. This is put into a separator and thoroughly shaken with ether, and allowed to stand at least 12 hours to insure complete separation of the ethereal and aqueous layers. This extraction is again repeated with a fresh lot of ether. We have generally found that more than two extractions are necessary. The ethereal solutions are combined, the ether removed by distillation and the residue dried, preferably in our experience *in vacuo*.

This process is easier and quicker than the sulphate of soda or calcium sulphate processes, or than the alcohol-ether processes. Fex found that for liver and kidney at any rate his process gave results agreeing with the sulphate of soda ether extraction process. He also found identical values, within errors of experiment, in parallel estimations, using this process and alcohol extraction according to the procedure of Schimidzu.

We are able to confirm fully Fex's claims as to the advantage of this method in tissue analysis, and that it avoids the displacement of the free to ester ratio which is sometimes noticed in tissues that have been air-dried or treated by the alcohol-ether process. We think its most important advantage is that the fresh tissues can be treated at once, so that possible autolytic or bacterial changes that may take place on standing for drying purposes are obviated.

Recently one of us has applied Fex's process to the analysis of blood plasma with very satisfactory results. When extracted by Bloor's process, or by drying with alcohol and subsequent alcohol and ether extraction, we have noticed occasionally that in the estimation by digitonin very troublesome colloidal suspensions were formed in washing the cholesterol digitonide, which made filtration difficult. This has not occurred in the extracts by Fex's process.

Inorganic esters of cholesterol.

In 1898 O. Hammarsten examined the bile of the great shark (*Scymnus borealis*) and found that it contained no cholesterol, and neither bilirubin nor urobilin [1898]. The mixture of sodium salts obtained by precipitating the bile acids with lead acetate and decomposing the precipitate with sodium carbonate, though responding strikingly to Pettenkofer's reaction, and the fluorescence test, contained no glycocholic or taurocholic acid. It consisted instead of the salts of at least two acids containing sulphur, which differed from taurocholic acid in losing the whole of their sulphur in the form of sulphuric acid in hydrolysis by acids. He named the two chief acids α- and β-scymnolsulphuric acid. The soluble α-scymnolsulphate was precipitated from a 3 % aqueous solution of the mixed salts by adding an equal volume of 40 % aqueous potash. The substance was white, containing 5·346 % of sulphur and produced a characteristic indigo blue colour when dissolved in 25 % hydrochloric acid: it was easily soluble in ethyl and methyl alcohol. By hydrolysis he isolated a crystalline substance, melting at 100°–101°, α-scymnol. This gave Pettenkofer's reaction, and the fluorescence test for cholic acid, and the same colour reaction with 25 % hydrochloric acid as the sulphate.

It resembled cholesterol in gving Schiff's test and the Borchard-Liebermann reaction with acetic anhydride and sulphuric acid.

He also described β-scymnol which he regarded as the next higher homologue of α-scymnol. He did not apparently isolate the substance in a sufficiently pure state to make his analysis by combustion of much value. He also obtained indication of the presence of such ethereal sulphates in human bile and in that of suckling animals. Probably Hammarsten had in hand some paired compound of cholesteryl ethereal sulphate and bile acid.

This led Mandel and Neuberg [1915] to prepare and examine ethereal sulphates of cholesterol. They succeeded in preparing cholesteryl potassium, sodium and ammonium sulphates by acting with chlorosulphonic acid on dry cholesterol, and neutralising the acid with the appropriate alkali.

We prepared both salts, following closely Mandel and Neuberg's directions. The salts were purified by repeated crystallisation from 80 % alcohol.

The sodium salt proved considerably more soluble in water than the potassium salt, and the ammonium salt was still more soluble. Mandel and Neuberg gave the melting point of their potassium salt as 235° with decomposition. Our preparation melted lower than this at 181°–183° with decomposition. They gave the melting point of the sodium salt as 163°–164° with decomposition. Our preparation melted at 163°–165°, with decomposition.

Sodium cholesteryl sulphate was practically unaffected by boiling with aqueous alkalis, and even with sodium ethoxide. It was, however, readily hydrolysed by acids. 0·0492 g. was boiled under a reflux for 4 hours with glacial acetic acid. The solution was then diluted with water and extracted with ether. The ethereal solution was freed from acid by washing with water and then with dilute alkali and evaporated to dryness. The residue, consisting of cholesterol and cholesterol acetate, was hydrolysed in the usual way with sodium ethoxide, diluted and extracted with ether. 0·1587 g. cholesteride was obtained corresponding to 0·0386 g. cholesterol. Hydrolysis was complete and the salt pure.

Potassium cholesteryl sulphate. This was also unacted on by aqueous alkalis. 0·0474 g. treated with acetic acid in the above manner yielded with digitonin 0·1486 g. of compound, equivalent to 0·0361 g. cholesterol; hydrolysis complete.

It is evident from these results that any cholesterol in the form of an alkali ethereal sulphate would be missed in any of the usual processes of extraction. Such cholesterol could only be detected and estimated by taking the material that had been extracted in the ordinary manner and boiling it with acid—say glacial acetic—for several hours. The acetic acid may then be distilled off, or neutralised and the fluid left extracted thoroughly with ether. The cholesterol extracted in this manner may, after hydrolysis of any ester of acetic acid, be estimated in the usual way. It is not excluded that cholesterol found in this manner might have been present in some other compound than an ethereal sulphate—for example, as an ethereal phosphate, which presum-

ably might have properties similar to an ethereal sulphate. As far as we are aware, however, no such phosphates have yet been described.

One of us has found traces of such cholesterol in the bile of the hippopotamus [Gardner, 1924]. We have also detected it in some specimens of human bile but not all, and also in normal human urine. An account of this work, however, we hope to make the subject of a future communication.

We take this opportunity of expressing our thanks to the Government Grant Committee of the Royal Society for a grant by which the digitonin was purchased.

REFERENCES.

Abderhalden and Kautzsch (1910). *Z. physiol. Chem.* **65**, 70.
Autenrieth and Funk (1913). *Münch. med. Woch.* **60**, 1243.
Bang (1911). Die Chemie u. Biochemie der Lipoide, **3**, 25 (Wiesbaden).
Bloor (1916). *J. Biol. Chem.* **24**, 227.
Czonka (1916). *J. Biol. Chem.* **24**, 431.
Erlandsen (1907). *Z. physiol. Chem.* **51**, 71.
Fex (1920). *Biochem. Z.* **104**, 82.
Fränkel and Elfer (1910). *Biochem. Z.* **28**, 330.
Fraser and Gardner (1910). *Proc. Roy. Soc. Lond.* B **82**, 559.
Gardner, Ellis and Lander (1910–1914). *Proc. Roy. Soc. Lond.*
Gardner and Fox (1921). *Biochem. J.* **15**, 3.
Gardner and Williams (1921). *Biochem. J.* **15**, 363.
Gardner (1921). *Biochem. J.* **15**, 244.
—— (1924). *Biochem. J.* **18**, 777.
Grigaut (1913). Le cycle de la cholesterinémie (Steinheil).
Hammarsten (1898). *Z. physiol. Chem.* **24**, 322.
Hürthle (1895). *Z. physiol. Chem.* **21**, 342.
Kumagawa and Suto (1908). *Biochem. Z.* **8**, 315.
Lapworth (1911). *J. Path. Bact.* **15**, 254.
Liebermann and Székely (1898). *Pflüger's Arch.* **72**, 360.
Marie (1896). *Liebig's Annalen*, **209**.
Mandel and Neuberg (1915). *Biochem. Z.* **71**, 186.
Mueller (1916). *J. Biol. Chem.* **25**, 549.
Rübow (1904). *Arch. exp. Path. Pharm.* **52**, 173.
Rosenheim (1906). *J. Physiol.* **34**, 104.
Rosenfeld (1900). *Centr. inn. Med.* **21**, 835.
Thaysen (1914). *Biochem. Z.* **62**, 89.
Weston and Kent (1917). *J. Biol. Chem.* **28**, 383.
Windaus (1910). *Z. physiol. Chem.* **65**, 110.

CXXXVIII. INVESTIGATIONS ON GELATIN. PART III. THE SEPARATION OF THE PRODUCTS OF HYDROLYSIS OF GELATIN BY THE CARBAMATE METHOD.

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Until the introduction by Dakin a few years ago of the butyl alcohol method for the isolation of certain of the hydrolysis products of the proteins, the only general process employed for the purpose was that of esterification originally elaborated by Emil Fischer. By Dakin's method a large proportion of the amino-acids could be separated in crystalline form without the necessity of converting them beforehand into their esters. When Dakin applied his method to the separation of the hydrolysis products of gelatin [1920] he met with certain difficulties which were not encountered in the case of the hydrolysis products of caseinogen. It was found, for example, that glycine and hydroxyproline were only very incompletely extracted from the mixture of the hydrolysis products by butyl alcohol, and in the case of the proteins which yield relatively large amounts of these acids on hydrolysis, the method of Dakin does not appear to be entirely satisfactory.

A few years ago Buston and Schryver [1921] showed that amino-acids could be readily separated from carbohydrates and other soluble products by precipitation in the form of their carbamates, a class of compounds originally described by Siegfried, when a solution containing them is treated in the presence of alcohol by barium hydroxide and carbon dioxide. It was found that by this method a very large proportion of the hydrolysis products of caseinogen and gelatin could be readily separated in a crystalline form without submitting them to the esterification treatment.

The objects of the work described in the present communication were, in the first instance, to determine the most suitable conditions under which the carbamates could be precipitated, and in the second instance, to ascertain whether the carbamate method could be adapted to the separation of the individual acids from one another. The investigation has been attended with a certain amount of success and the main outlines are now clear, although a considerable amount of detailed work is still necessary, and is now being carried out in this laboratory to make the process a more complete one.

General method.

The proteins are hydrolysed in the usual manner with dilute sulphuric acid, and the hydrolysis mixture is then treated with barium hydroxide until the solution is just acid with Congo red. The barium sulphate is then filtered off, and more barium hydroxide is added to the filtrate and a second precipitation of barium sulphate is produced. This second precipitate was found to contain appreciable quantities of nitrogen, and on isolating the amino-acid to which it belonged by the method described below, *i*-aspartic acid was obtained.

By adding alcohol (3 vols.) to the filtrate from the second barium sulphate precipitate, a crystalline precipitate was formed, which was found to consist of the barium salts of the dicarboxylic acids (glutamic and aspartic acids only, hydroxyglutamic acid not being present amongst the hydrolysis products of gelatin). The precipitation of the dicarboxylic acids at this stage appears to be complete, no trace of them having been found in any later fraction.

The carbamates were then precipitated in the filtrate by alternate treatment of the ice-cold alcoholic liquid with barium hydroxide and carbon dioxide by the method described in some detail below. The filtrate from the barium carbamates was then concentrated and recarbamated, and the carbamation process repeated until practically no nitrogen was brought down by the treatment of ice-cold alcoholic solutions with barium hydroxide and carbon dioxide. The final filtrate from the carbamates contained generally 6–7 % of the total nitrogen and was almost free from amino-nitrogen. This nitrogen is almost entirely in the form of either proline or prolyl-proline. The exact form has not yet been determined.

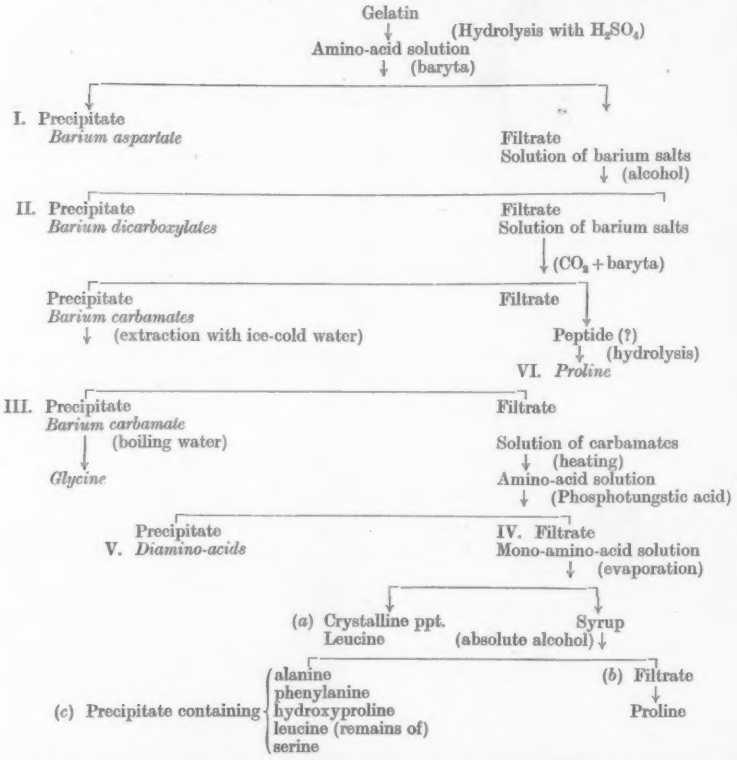
The carbamate fractions were collected together (for details, see below) and when dried, extracted with ice-cold water. This extracts everything except the barium carbamate of glycine. When this is decomposed with boiling water, almost pure glycine separates from the aqueous solution after filtration from the barium carbamate. No glycine has been found in any other fraction.

The soluble carbamates were decomposed by boiling their solution with water. A fraction was thereby obtained which contains also the diamino-acids. These, it has been found by separate experiment, are readily precipitated as carbamates under the conditions generally used. They can be separated as phosphotungstates in the usual way. On evaporating the soluble carbamate fraction after separation of the bases, the greater part of the leucine separated in a nearly pure condition. The filtrate from the leucine, after concentration, was thrown into alcohol, and a crystalline precipitate was produced. The alcoholic filtrate was then concentrated, the residue taken up by absolute alcohol, and filtered from the small amount of precipitated matter, which was added to the precipitate just mentioned. The alcoholic solution was repeatedly evaporated to dryness, and taken up with alcohol until a product was obtained which completely dissolved in absolute alcohol, producing a solution which no

longer formed a crystalline precipitate after prolonged standing in an ice-chest. The only amino-acid obtained from this solution, which contained practically no amino-nitrogen, was proline. It was purified by conversion into the ethyl ester which all distilled within one or two degrees when submitted to fractionation. The amino-acids precipitated by alcohol in obtaining the proline fraction include the remains of the leucine, valine, serine and hydroxyproline. The last named can be estimated by determining the amount of non-amino-nitrogen in the fraction. The phenylalanine can be readily isolated by converting the fraction into the esters and extracting the aqueous suspension by ether in the usual way.

The separation of the amino-acids in this fraction forms the least satisfactory part of the general process, and researches are still being carried out with a view to evolving a more satisfactory process for dealing with them.

Diagram of the Separation of the Hydrolysis Products of Gelatin.



The carbamate method for separating the hydrolysis products of gelatin has been employed in experiments where several hundred grams, and also

where only 10 g. of gelatin have been hydrolysed. Even when only 10 g. of material are used for investigation, fairly accurate estimations of the following amino-acids are possible, viz. glycine, aspartic and glutamic acids, the diamino-acids (in which fraction, of course, the amounts of arginine, histidine, and lysine can be estimated in the usual manner) proline, and hydroxyproline; and the nitrogen of the non-carbamating fraction can also be estimated.

It is possible that a good routine method for the analysis of the proteins when only small amounts of material are available may be based on the carbamate method, but minor details have still to be worked out, and attempts are now being made in the laboratory to improve the process. The chief advantage over other methods seems to consist in the ease with which the glycine, dicarboxylic acids and the proline can be separated in a pure form.

The general scheme of separation is outlined in the table p. 1072.

The following are the quantitative results obtained from a large scale and a small scale experiment. In the large scale experiment, the liquid was not sufficiently diluted when the bases were precipitated by phosphotungstic acid, consequently the diamino-nitrogen found was too high, and the results are not therefore quoted here. Otherwise fair agreement was found between the results of the large scale experiment (with 900 g. gelatin) and the small scale experiment (with 10 g.).

Results in percentages of total N.

Large scale experiment		Small scale experiment		
Glutamic acid	3·2 %	Fraction 1.	Aspartic acid and humin N	4·49 %
Aspartic „	5·6	„ 2.	Dicarboxylic acids*	6·50
Proline ...	14·8	„ 3.	Glycine	17·44
Hydroxyproline	11·25	„ 4 *a*.	Leucine, serine, hydroxyproline, alanine	17·26
Glycine ...	18·2	„ 4 *b*	Proline	12·06
		„ 5.	Diamino-acids	26·67
		„ 6.	Non-carbamating fraction	6·93

* Does not include the aspartic acid of Fraction 1.

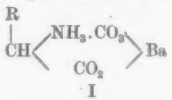
The results in the small scale experiment on addition give 91·3 % of the total nitrogen. To this must be added 2 % to account for the amide nitrogen which is estimated separately. It was found subsequently that the barium carbonate obtained after decomposition of the carbamates always contained a small amount of nitrogen. This, unfortunately, was not estimated in the above experiment. If this had been done, the total N accounted for would have been higher than 93 %.

No particular importance is to be attached to these values as absolute quantities. Subsequent researches on gelatin, which will form the subject of later communications, show that the hydrolysis products of gelatin can vary appreciably, according to the method employed for the preparation (even when different samples are made from the same precursor) and the subsequent treatment.

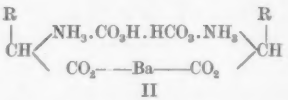
Some experimental details.

General method of carbamation.

It seemed possible that two types of carbamates could be produced, according to whether the solution of the amino-acids were treated in the first instance with carbon dioxide or with barium hydroxide. In the former case barium salts of the general formula I.



I



II

might be expected, whereas in the latter case salts of the type II might be expected to be formed, from which, on addition of a further amount of barium hydroxide, another barium salt would be produced, in which the two hydrogen atoms of the CO_2H groups would be replaced by the metal. An extended series of experiments was therefore carried out with the object of determining the conditions under which the amino-acids could be precipitated with the smallest amount of barium carbonate. It was found that large amounts of nitrogen were only precipitated when the ice-cold aqueous-alcoholic solution (two volumes of alcohol to one of water) was never allowed to become acid to phenolphthalein. The process of precipitation can be divided into three stages. (*a*) Recrystallised, moist or finely powdered dry barium hydroxide is added little by little to the aqueous-alcoholic solution kept cool by ice with continuous stirring until no further quantity dissolves. Phenolphthalein is added in sufficient amount to produce a bright pink colour. (*b*) Carbon dioxide is then passed in until the pink colour just fades. (*c*) Recrystallised moist barium hydroxide is then added again, with continuous stirring, until no more dissolves. A thick gelatinous precipitate is thereby produced which contains the carbamates contaminated with relatively small amounts of carbonate. The barium carbamates appear to belong to the second type of salts described above.

Attempts have been made to form carbamates of metals other than barium. The calcium salts are similar in most respects to the barium salts, but the latter are more conveniently prepared owing to the greater solubility of barium, as compared with calcium, hydroxide. The lead salts are difficult to prepare by the direct action of lead hydroxide. The copper salts are soluble. Separate experiments with the mixed diamino-acids show that these are as readily precipitated as the carbamates.

The method may be best illustrated by a description of a complete experiment. 900 g. of gelatin were soaked in five times the weight of water for one day. The mixture was then heated to boiling point on a water-bath and whilst still hot added to a boiling solution of sulphuric acid of such strength that the mixture contained 25 % sulphuric acid (for reasons of employing this method see Knaggs [1923]). Heating was continued for 30 hours. After dilution to

5400 cc. barium hydroxide was added until the mixture was just distinctly acid to Congo red, and the barium sulphate was then filtered off. To the filtrate more barium hydroxide was added in quantity somewhat more than sufficient to convert all the amino-acids into the barium salts, which amount was determined by a formaldehyde titration of a small part of the liquid. The barium sulphate thereby precipitated contains nitrogen; this is nitrogen of *i*-aspartic acid which gives an insoluble barium salt (Fraction 1 of the table, p. 1072). The filtrate from the barium sulphate containing the barium aspartate was concentrated to a volume of 3 litres and mixed with twice the volume of 95 % alcohol, and the mixture was mechanically stirred and then allowed to stand for two days. The barium salts of the dicarboxylic acids separated in a crystalline form.

After filtering off this precipitate, the liquid was cooled with ice, phenolphthalein was added, and then finely powdered dry, or freshly recrystallised moist barium hydroxide, until undissolved crystals remained. The mixture was vigorously stirred the whole time by a mechanical stirrer, the operation being carried out in 10-litre enamelled buckets. Carbon dioxide was then passed in with continual stirring until the phenolphthalein was nearly decolorised; more baryta was then added (until there was an undissolved excess) and the stirring was continued for about two hours and carbon dioxide again passed in. After this alternate treatment with barium hydroxide and carbon dioxide had been repeated twice and excess of baryta added, the gelatinous precipitate was filtered off (precipitate I), first on large ordinary filters, from which it was removed after the greater part of the liquid had separated to a Buchner funnel, when the filtration was completed by suction. The filtrate was again treated alternately twice with baryta and carbon dioxide and a second precipitate was obtained (precipitate II). A third fractional precipitate was obtained in a similar way. These three precipitates were washed by grinding with aqueous alcohol (2 vols. 95 % alcohol:1 vol. water), sucked as dry as possible on a Buchner funnel, then washed with graded strengths of alcohol and finally with ether and air dried. The aqueous alcoholic washings were added to the filtrate from precipitate III, and this liquid was then treated with carbon dioxide to precipitate excess of baryta, and after filtration of the precipitated carbonate, evaporated under diminished pressure to 1 litre. After addition of 2 litres of 95 % alcohol to this liquid and cooling with ice, carbamation was carried out three times in the manner already described. In this way a fourth precipitate was obtained. The following table indicates the course of the precipitation of the amino-acids.

Precipitate	Weight of fraction g.	% of N in fraction	% of N precipitated in fraction
I	1350	4·9	66·15
II	226	3·4	7·46
III	136	4·5	6·12
IV	147	2·2	3·23

In the last column the percentage of the nitrogen in the liquid which was

carbamated (after separation of the dicarboxylates) precipitated in each fraction is indicated. All these precipitates after drying were obtained in the form of fine powders. Further precipitation brings down only very small amounts of nitrogen.

The examination of the various fractions.

The various fractions are designated by the numbers given in the general scheme of separation (on p. 1072).

Fraction I. The greater part of the sulphuric acid used in the hydrolysis is first precipitated by adding baryta, only so much baryta being added as to leave the liquid still markedly acid to phenolphthalein. The first barium sulphate precipitate contains only small quantities of nitrogen. A second fraction of barium sulphate is then produced by adding more than sufficient baryta to combine with all the carboxyl groups (the amount necessary being determined by a formalin titration). The second precipitate contains appreciable amounts of nitrogen. In one experiment when 900 g. of gelatin were hydrolysed this amounted to 1·2 g. It was boiled with dilute sulphuric acid, and from the extract thus obtained the sulphuric acid was precipitated quantitatively by barium hydroxide. The filtrate from the barium sulphate was concentrated to a syrup, which was then thrown into absolute alcohol. A granular precipitate weighing 12·14 g. was obtained. It was purified by reprecipitation as barium salt by the addition of barium hydroxide to its aqueous solution. The product obtained by regeneration of the acid from the barium salt contained 10·44 % N (calculated for $C_4H_7O_4N$ 10·5 %). The substance agreed in its properties with *i*-aspartic acid, which is known to give a barium salt which is almost insoluble in water.

Fraction II. The crystalline alcoholic precipitate produced by the addition of alcohol was filtered off, and the free acids generated therefrom. They consisted of aspartic and glutamic acids, but no trace of hydroxyglutamic acid was found. The two former acids could be readily separated from one another, the glutamic acid being separated in the form of its hydrochloride.

Fraction III. The various fractions of the carbamate precipitates described above, which contain considerable amounts of barium carbonate, weighing altogether when dried 1840 g., were mixed with 5 litres of ice-cold water, with which they were mechanically stirred for two hours in an enamelled bucket surrounded with ice. After filtration the precipitate remaining undissolved was again extracted with ice-cold water (2·5 litres), and this extraction was repeated after filtration twice more with quantities of 1·5 and 1 litres of water. The aqueous solution contained the soluble carbamates. The insoluble fraction was decomposed by blowing steam through a suspension in hot water for about two hours. The carbamate was thereby decomposed into barium carbonate and free amino-acid. The solution of the latter was filtered off and concentrated. Crystals separated out. Various fractions were obtained and the mother-liquor from the last crop was thrown into absolute alcohol. A granular pre-

cipitate was thereby produced. The nitrogen was estimated in all the crops and found to be about 17·9 %. Nothing but glycine could be obtained from this fraction, nor could any glycine be isolated from other fractions; all attempts to isolate an insoluble barium carbamate from them led to a negative result. No precipitate was produced by phosphotungstic acid (although such a precipitate has been obtained in the corresponding fraction from other kinds of gelatin, S.B.S.). The glycine was further characterised by its conversion into the hydrochloride of the ethyl ester.

Fraction IV. The soluble barium carbamates obtained by the method described above were decomposed by boiling this solution. The barium carbonate thus precipitated was filtered off. The filtrates and washings were diluted so that the solution contained about 2 % amino-acids, and the diamino-acids were precipitated from this by phosphotungstic and sulphuric acids in the usual manner, this yielding *Fraction V.* (This was not further examined.) After excess of phosphotungstic acid and sulphuric acid, etc., had been separated in the usual manner, the solution freed from diamino-acids was concentrated *in vacuo.* Crystals separated. The first fraction weighed 13·01 g. and contained 10·06 % N. This consisted of almost pure leucine. The filtrate from this was concentrated to a small bulk and then thrown into absolute alcohol. A granular precipitate was obtained, weighing, after washing with alcohol and drying, 158 g.; it contained 11·9 % N of which 9·4 % was amino N, as determined by the method of Van Slyke. This sub-fraction consists of the remainder of the leucine which had not separated out, phenylalanine, alanine and serine. The amount of hydroxyproline could be determined indirectly by estimating the amount of non-amino N in the fraction. Investigations are still taking place on the separation of the amino-acids in this fraction. It was esterified in the ordinary way, and the free esters were obtained from the hydrochlorides. The conversion of the hydrochlorides into the free esters by Foreman's method [1919] was not found to be satisfactory, as the decomposition of the hydrochlorides in chloroform solution by barium oxide was very slow and incomplete. This may be due to the presence of hydroxyproline, as a similar difficulty with the corresponding fraction from caseinogen, which contains only a little hydroxyproline, was not experienced. In the case of gelatin, the decomposition was effected by sodium ethoxide in alcoholic solution. From the mixture of esters thus obtained, that of phenylalanine was readily obtained by extracting the aqueous suspension with ether.

The alcoholic filtrate from the precipitation of the amino-acids after separation of the leucine contains the proline. The alcoholic solution was evaporated down, and the residue was taken up with absolute alcohol and this solution filtered, the filtrate was evaporated and taken up with absolute alcohol, and these processes were repeated until a product was obtained which was completely soluble in absolute alcohol, giving a solution which yielded no deposit after prolonged standing in an ice-chest. This solution was almost free from amino-nitrogen. The ester hydrochloride of the alcohol-soluble portion

was prepared in the usual manner. The conversion into free ester was readily effected by Foreman's method. The ester had a nearly constant B.P. of 80° at 15 mm.: and was found to be the ester of pure proline, which was identified by the estimation of copper and nitrogen in the copper salt, and by conversion into the hydantoin derivative.

Fraction VI. This fraction, which formed the filtrate from the carbamates, was obtained in the form of a thick syrup by concentration *in vacuo*. This was dissolved in water and hydrolysed for 12 hours with 25 % sulphuric acid. After the quantitative separation of the acid by barium hydroxide and concentration a syrup was obtained which upon throwing into alcohol gave only a slight precipitate. The alcoholic solution was again concentrated, and the resultant syrup again hydrolysed with 25 % acid, and the processes just described repeated. A syrup was finally obtained which was completely soluble in alcohol. After concentration of the alcoholic solution a product was obtained which formed on standing a semi-crystalline mass. By means of cupric hydroxide a crystalline salt soluble in alcohol was obtained, with all the properties of the copper salt of proline. The copper and nitrogen were determined in this, and the results confirmed the assumption that the substance was the salt of proline.

The examination of this fraction has not been completed. The results do not indicate definitely whether it contained the prolyl-proline of Dakin [1920] or simply proline which had escaped precipitation by the carbamate method.

This work was carried out during the tenure of a Studentship of the Department of Scientific and Industrial Research by one of the authors.

REFERENCES.

Buston and Schryver (1921). *Biochem. J.* **15**, 636.
Dakin (1920). *J. Biol. Chem.* **44**, 499.
Foreman (1919). *Biochem. J.* **13**, 378.
Knaggs (1923). *Biochem. J.* **17**, 488.

CXXXIX. INVESTIGATIONS ON GELATIN. PART IV. THE PURIFICATION OF GELATIN BY FLOCCULATION IN AN ELECTRIC FIELD.

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Report to the Adhesives Research Committee of the Department of Scientific and Industrial Research.

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Introduction.

In a previous paper a method of purifying gelatin by separation from aqueous solution was described [Knaggs, Manning and Schryver, 1923]. In the present paper an alternative method is indicated. In carrying out a process such as this, it is essential to distinguish between the impurities due to the tissues from which the gelatin is derived, and those due to its thermal degradation. Most of the experiments, therefore, were carried out with a gelatin prepared in the laboratory from bone.

Preparation of Material.

Ossein, which was kindly supplied by Mr W. K. Beveridge, of Messrs Nelson Dale and Co., was used. The pieces of this material were approximately $\frac{1}{4}$ inch size. It was treated with 10 % brine and 0·5 % hydrochloric acid for 24 hours and well washed in running water to remove all the inorganic salts as far as possible. The ossein was then treated with 0·2 % sodium hydroxide solution, 100 g. of dry ossein to 1 litre of the alkali, for 60 days. At frequent intervals, the caustic soda solution was decanted off and a fresh batch added.

The prolonged treatment with 0·2 % caustic soda was found necessary to remove the last traces of the substances soluble in alkali present in the tissue, such as chondroitinsulphuric acid. The rate at which the latter substance was removed was followed by treating 100 g. of dry ossein with 1 litre of 0·2 % caustic soda; 25 cc. of the liquid were removed each day, and the amount of nitrogen determined by the Kjeldahl method. It will be seen from the following results that the nitrogen becomes nearly constant after eight days. Yet if the caustic soda solution is removed and a fresh quantity added, more of the chondroitinsulphuric acid is extracted; the solution gave a precipitate with 1 % acetic acid, showing the presence of a mucin, or some similar substance. It would seem from the results that the chondroitinsulphuric acid is in chemical combination with some other colloid present in the ossein and the above numbers give an indication of the rate at which such a complex is split up by dilute alkali.

Time in days	Mg. of N per 100 cc. NaOH solution
1	16·8
2	20·6
5	24·92
6	25·48
7	26·32
8	26·88
9	27·16
10	27·30

The ossein was therefore left in contact with the 0·2 % caustic soda solution for 60 days. Every third day the solution was changed, and tested for the presence of chondroitin by dilute acetic acid.

It was only after 51 days that no turbidity was observed when the soda solution was acidified.

This fact was also confirmed by the Hausmann numbers of gelatins which were extracted from ossein treated for varying lengths of time with 0·2 % caustic soda.

The following is the table, and the Hausmann numbers of a sample of a high-grade commercial bone gelatin are included for comparison.

Number of days ossein was treated with 0·2 % NaOH	Amide N	Humin N	Diamino N	Diamino N and humin N
1 day 1st extraction	3·3	1·3	19·9	21·2
2nd „	3·3	1·5	20·0	21·5
7 days	3·2	1·8	21·4	23·2
14 days 1st extraction	2·59	1·85	21·8	23·65
2nd „	3·0	·87	22·0	22·87
14 days 2nd „	2·48	1·6	22·8	24·4
(The gelatin was washed with *N*/1000 HCl and water for 10 days)				
30 days	1·76	1·48	23·4	24·88
60 days	1·54	1·28	24·09	25·37
Commercial bone gelatin	2·04	3·93	23·06	26·99
Commercial bone gelatin purified by washing with *N*/1000 HCl and water 10 days	1·7	3·35	23·05	26·40

Gelatin extracted from ossein which has had no treatment with soda has a percentage of amide nitrogen of 3·76. By treating with caustic soda the percentage of amide nitrogen is progressively reduced to 1·54 after 60 days, which is the minimum value which has been obtained for bone gelatin.

In the same way the percentage of diamino-nitrogen is increased from 19·9 after one day's treatment to 24·09 after 60 days' treatment with 0·2 % NaOH.

As the caustic soda extracts from the ossein chondroitinsulphuric acid, some of this compound was isolated from the alkaline extract by acidifying with acetic acid. The precipitate was collected and washed with dilute acetic acid on the filter. The Hausmann numbers were as follows:

Percentage of	amide nitrogen	7·67
„	humin „	4·60
„	diamino „	15·48

This protein has a high percentage of amide N 7·67 and a low percentage of diamino N 15·48, and its presence in gelatin would appreciably alter the Hausmann numbers, as above results indicate.

The percentage of amide N in a sample of gelatin would be a good criterion as to its purity. It would give no indication of the products of thermal decomposition, but the presence and approximate amount of other protein impurities could be detected fairly accurately.

In the following experiments on flocculation a gelatin was used which was freed as far as possible from organic nitrogenous impurities such as chondroitin. The gelatin was extracted from the ossein at 80° to ensure that the percentage of heat degradation product was as low as possible.

The method of purifying gelatin by passing a current through a 20 % jelly [Knaggs, Manning and Schryver, 1923] was only suitable for removing soluble inorganic salts. The protein impurities and insoluble inorganic salts were only partly removed.

This was proved to be the case by submitting a gelatin known to contain these impurities to this process. The gelatin used was extracted from ossein treated for only 5 days with 0·2 % caustic soda. The gelatin was washed with *N*/1000 HCl and then with frequent changes of water for 10 days. The percentage of ash in the washed sample was found.

A 20 % gel of the gelatin was made up, and electrolysed, and after several days the gelatin was examined quantitatively.

The Hausmann numbers of each fraction are given below.

	Amide N	Humin N	Diamino N	Diamino N and humin N
A. Gelatin from ossein treated for 5 days with 0·2 % caustic soda	3·26	2·53	21·76	24·29
A was washed with *N*/1000 hydrochloric acid and then with water for 10 days	2·83	2·41	22·00	24·41
The washed gelatin was electrolysed as a 20 % gel	2·98	2·54	21·95	24·49

The process of washing removed some of the organic impurities, shown by the change in the percentage of amide N from 3·26 to 2·83. The electrolysis did not further reduce the values, for the Hausmann numbers of the washed gelatin and electrolysed gelatin are practically the same.

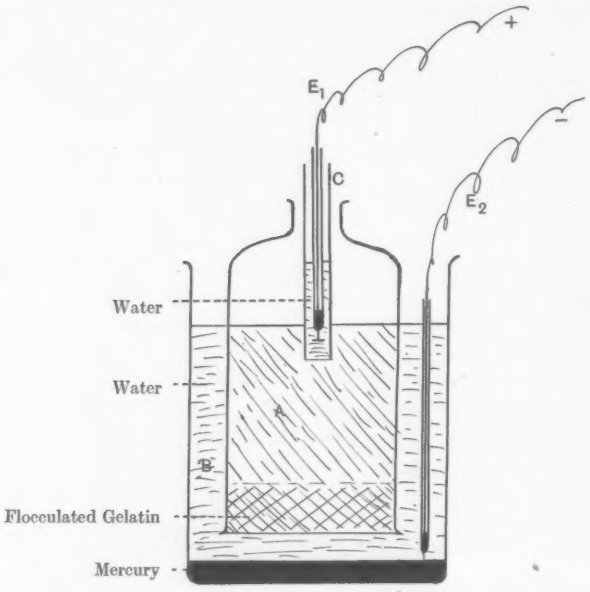
During electrolysis of gelatins obtained from ossein which had not been treated for a sufficient length of time by alkali, the jelly became very turbid, but this turbidity could not be removed by filtration of the hot solution through paper pulp. This was probably due to the chondroitinsulphuric acid being split up under the influence of the current into chondroitin and sulphuric acid, the chondroitin being held in suspension in the gel. The Hausmann numbers would not indicate such a change as the percentages of each group are calculated from the total nitrogen present in the protein.

The ash in the washed gelatin amounted to 0·35 % and consisted almost entirely of calcium phosphate. After electrolysis this value was 0·32 % and the gelatin could readily be "recrystallised" from a 1 % solution.

In carrying out the experiment on purification by flocculation, it was found advisable, therefore, to use a gelatin made as free as possible from chondroitin, etc., by prolonged treatment of the ossein with alkali, and to free it, finally, from electrolytes by the electrolysis of a 20 % gel.

Flocculation of gelatin from electrolytes and tissue impurities.

Method of working. The apparatus which was used is shown graphically in the accompanying figure.



A is a bell-jar internal diameter 7·8 cm. and height 30 cm. across the bottom of which is stretched muslin firmly bound by means of string. The muslin is rendered semi-permeable by a coating of collodion.

B is a bucket or glass vessel, the base of which is covered with a layer of mercury to act as cathode E_2. *B* is filled with a known volume of water and the base of *A* dips into the water as shown.

C is a piece of glass tube internal diameter 1 inch and length 8 inches, the end which dips into the liquid being also closed by a collodion membrane. The tube *C* is filled with water and the anode E_1 is immersed in the liquid. By separating the anode in this way with a membrane, any acid which is liberated during electrolysis is isolated from the liquid in *A*.

In the experiment about to be described, a gelatin was employed which had been prepared from an ossein which had been treated for 6 weeks with 0·2 % NaOH solution. 6 litres of a 2 % solution were placed in *A*. The poles were 30 cm. apart and a P.D. of 150 volts was established between them. In this experiment the electrolytes had not been completely removed by a preliminary electrolysis of the gel.

The current was passed for several days. When all the electrolytes were removed, the gelatin solution became cloudy, and the protein after a time was precipitated in *A* as a white flocculent mass. After 5 days the solution became quite clear, and the precipitated gel was very similar to one which had been recrystallised from water.

The distribution of the nitrogen in the various layers was found to be as follows:

Round anode	negligible
Supernatant liquid	17·38 %
Precipitated gelatin	64·33 %
Diffused through membrane to cathode *B*	18·18 %

The strength of the precipitated gelatin was found to be 7·5 %; and the mg. of N per 100 cc. of the supernatant liquid, and the liquid round the cathode, were 34·12 and 32·1 respectively.

Experiments were carried out to determine the rate at which the gelatin was precipitated.

The same sample of gelatin as above was used, but it was first washed with water for 4 days, and the last trace of ionisable salts removed by electrolysing a 20 % gel. Experience showed that purification by electric flocculation can only be conveniently carried out with a gelatin freed from chondroitin by the method described above, and subsequently purified by washing and the electrolysis of a strong solution.

The apparatus was the same as described above, except that the tube *C* was removed so that the anode dipped directly into the gelatin solution. At known intervals of time 50 cc. of the supernatant liquid in *A* and the cathode liquid in *B* were pipetted out, and the amount of nitrogen determined by Kjeldahl's method.

The current was allowed to pass until the value mg. N/100 cc. in both the supernatant liquid and cathode liquid became constant.

The following table gives the readings for the first and second flocculations:

	First flocculation			Second flocculation	
Time in hrs	Supernatant liquid mg. N/100 cc.	Cathode liquid mg. N/100 cc.	Time in hrs	Supernatant liquid mg. N/100 cc.	Cathode liquid mg. N/100 cc.
0	240·8	0	0	207·2	0
24	97·4	3·11	24	53·34	2·1
54	42·98	2·90	48	26·35	2·55
100	29·77	3·11	70	13·31	2·27
192	15·15	3·11	120·5	11·05	2·55
220	14·8	3·0	145·5	3·97	1·71
244	15·1	2·9	169·0	5·38	1·98
268	14·7	3·20	193·0	5·52	1·70
			217	5·67	1·98
			234	5·52	2·27
			286·5	5·52	2·58

The gelatin which was precipitated in the first flocculation was redissolved and reflocculated by the electric current. The readings under "Second flocculation" refer to this.

First flocculation. After the current has been passing for 200 hours the nitrogen remaining in the supernatant liquid becomes constant at 15 mg., but in the liquid round the cathode this value becomes constant within the first 24 hours at 3·0 mg. Therefore the portion which diffuses through the parchment to the cathode must be of low molecular weight and carry a relatively high electric charge.

Second flocculation. The readings are very similar to those of the first flocculation except that when the amount of nitrogen becomes constant in the supernatant liquid, the value is 5·5 mg., which is much lower. The liquid round the cathode reaches a constant nitrogen value within the first 24 hours of 2·0 mg. per 100 cc.

A third flocculation was carried out, and it was found that when equilibrium was established nitrogen remaining in the supernatant liquid was 6·3 mg. per 100 cc., and that which diffused through the membrane to the cathode was 1·1 mg. per 100 cc.

The quantity of nitrogen in the supernatant liquid in the third flocculation is higher than in the second flocculation, and the precipitated gelatin when "recrystallised" from water did not separate properly on standing, but always gave a turbid liquid. The turbidity could be readily removed by electrolysing the solution. The only way this could happen would be by the solution containing a trace of electrolyte. The experiments described above were carried out using an enamelled bucket to contain the cathode liquid. Under the influence of an electric current some of the salts from the enamel would most likely find their way into the flocculated colloid. The experiments were therefore repeated using glass vessels, and the following results show the above consideration to be correct:

Experiment carried out in glass vessels	mg. N remaining in the supernatant liquid when constant
Second flocculation	5·09
Third „	4·53
Fourth „	3·41

The value 3·4 mg. N per 100 cc. appears to be a constant for the particular gelatin, for even after seven flocculations the value does not alter.

Summary.

1. It is shown that the Hausmann numbers of bone gelatin can be influenced by the amount of chondroitinsulphuric acid present. The compound may be removed from ossein by treating it for 60 days with 0·2 % caustic soda.

2. Gelatin may be freed from the products of thermal degradation by flocculating several times in an electric field a 2 % solution of gelatin which is free from electrolytes, chondroitin, etc.

3. After a certain number of flocculations, under the conditions of experiment specified, the amount of nitrogen remaining in the supernatant fluid becomes constant, and is not diminished on further flocculation.

Reference.

Knaggs, Manning and Schryver (1923). *Biochem. J.* **17**, 473.

CXL. INVESTIGATIONS ON GELATIN. PART V. THE PROPERTIES OF A GELATIN PURIFIED BY FLOCCULATION IN AN ELECTRIC FIELD.

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In the course of some of the experiments on the "recrystallisation" of gelatin, solutions were obtained occasionally which refused to separate on standing. At first it was thought that a stage of purification had been reached where a gelatin could form a stable suspension in water and that the presence of a trace of some impurity was necessary for precipitation.

But further investigation showed that the non-precipitation was actually due to the introduction of a small quantity of electrolyte as impurity, most probably ammonia due to a very slight hydrolysis of the gelatin. Traces of electrolyte present in the distilled water or dissolved from the glass vessels used may also play a part.

Such suspensions could, however, be completely and rapidly precipitated by placing them in an electric field. This method of precipitation has already been described [Knaggs and Schryver, 1924].

(The gel can be removed, rinsed with cold water and dried down. *This gelatin will again separate from a* 1 % *solution on cooling.*)

The upper liquid after electrical precipitation is perfectly clear and contains only traces of nitrogenous material. Thus analyses of several such preparations gave:

N content = 2·0–2·4 mg./100 cc. when upper pole +[1]
„ = 1·4–1·6 mg./100 cc. „ „ „ −.

In these experiments platinum was used for both the cathode and anode.

A sample of bone gelatin which, though ash free, yet contained breakdown products as an impurity, gave the following results:

First precipitation	9·1 mg./100 cc.
Second „	3·5 mg./100 cc.
Third „	2·7 mg./100 cc.

These are the N content of the clear liquid at + pole.

(*Note.* This particular sample is being further investigated.)

[1] These numbers appear to vary slightly in different samples of gelatin and probably also with the current density used and other factors.

These low values of the N content of the clear solutions obtained after electrical precipitation reopened the question of the solubility of gelatin in water. The solutions obtained after ordinary flocculation of a solution of ash-free gelatin are usually very slightly opalescent. Occasionally they appear quite clear at first but invariably on standing develop this slight opalescence. As described in a previous report, these solutions contain always about 10 mg. N per 100 cc. It was decided to investigate the behaviour of these solutions in an electric field.

When placed in a U-tube and subjected to an E.M.F. the first action is to produce cataphoresis, the particles moving towards the anode. They are therefore negatively charged. The liquid round the cathode becomes perfectly clear and the moving boundary between clear liquid and the opalescent suspension is very marked. Approximately the particles move at the rate of $\frac{1}{2}$ cm. an hour under a potential gradient of 10 volts/cm. After a few hours, however, coagulation of the particles is noticeable, and gradually the gelatin is precipitated to the bottom of the U-tube, leaving the liquid in the upper part of the tube quite clear round both electrodes.

Further experiments were carried out in the larger apparatus described in the preceding paper (p. 1082) and the N content of the clear liquid determined. In every case it fell to between 1 and 2 mg./100 cc. after precipitation of the gelatin and was always a little higher round the + than round the − pole. It may here be recorded as supplementing previous observations [Knaggs, Manning and Schryver, 1923, Section IV], that if such a dilute solution is concentrated under reduced pressure at 40° to one-fifth of its bulk, the resulting solution flocculates on cooling with separation of the solid hydrated gelatin, *if the dilute solution is concentrated within* 12 *hours from being made up.* If it is concentrated after being allowed to stand three or more days this flocculation does not occur.

The gelatin remaining in solution after the flocculation of dilute solutions of ash-free gelatin is therefore present as a negatively charged suspension. In other words, the gelatin is peptised to the extent of 10 mg. N/100 cc. by adsorbed negative ions, probably OH ions, present in the water. The extreme sensitiveness of gelatin to OH ions, as shown by experiments described below, supports this conclusion.

The very minute amount of nitrogenous material remaining in solution after electrical precipitation of these suspensions is probably due to slight decomposition of gelatin at the two poles. There would probably be greater decomposition due to oxidation at the anode than to reduction at the cathode. The experimental results bear out this supposition. It may be noted here that the current used in the electro-dialysis or electro-precipitation of the purified gelatin does not exceed 10 milliamps.

Effect of addition of acid, alkali and neutral salts to solutions of purified gelatin.

A roughly quantitative comparison was made of the effect of acid, alkali and neutral salts in producing a clear gel or sol from dilute solutions of the purified gelatin.

The concentration of gelatin used was 0·5 %. On cooling this solution the usual flocculent precipitate and slightly opalescent supernatant liquid were obtained. Quantities of 100 cc. of this solution were transferred to 100 cc. flasks and the requisite quantity of acid, alkali or neutral salt added. The solutions were allowed to cool and observations were made after standing 3 days at room temperature (13° to 15°).

Ammonium hydroxide.

Concentration	Appearance of solution
0·00002 *N*	Flocculent precipitate, but the supernatant liquid more turbid than in water alone
0·00005 *N*	No separation of solid even on centrifuging. Solution completely peptised to a stable translucent gel
0·0001 *N* and higher concentrations up to	Solutions transmit more light with increasing concentration of alkali. All these solutions form weak gels.
0·001 *N*	Transparent gel

Hydrochloric acid.

Concentration	Appearance of solution
0·00001 *N*	Flocculent precipitate, but the supernatant liquid more turbid than in water alone
0·00002 *N*	Flocculent precipitate, but the supernatant liquid yet more turbid
0·00005 *N*	No separation of solid; a slightly translucent gel is formed
0·0001 *N*	No precipitation; opalescent gel
0·0005 *N*	Quite clear gel

Potassium chloride or barium chloride.

Concentration	Appearance of solution
0·0002 *N*	Flocculent precipitate; separation almost as complete as with water alone
0·001 *N*	Solid separated, but not so readily. Supernatant liquid slightly more turbid
0·005 *N*	Very weak opaque gel formed. On standing extensive syneresis occurred
0·025 *N*	Stable gel, but still rather opaque.
0·1 *N*	Nearly clear gel

It will be observed that only very small concentrations of acid or alkali are necessary to produce a clear and stable gel. On the other hand, a relatively large concentration of neutral salt is necessary to produce the same effect. In these respects, gelatin behaves like a typical globulin. If to a solution just sufficient acid is added to produce a clear stable gel, and if this gel is remelted at 35° and neutralised with an exactly equivalent quantity of alkali, on cooling, the gel again becomes white and opaque, and separates on standing into two phases. The salt formed by neutralisation of the acid is in concentration considerably below that necessary to produce a clear and stable gel. A similar phenomenon, though very much less marked, can be observed with some good commercial gelatins. By the careful addition of small quantities of acid a point

may be observed at which the gel exhibits a maximum turbidity (the iso-electric point). Generally however a commercial gelatin from which the inorganic impurities have not been removed gives a clear and stable gel even when brought to the iso-electric point by the addition of acid. That is to say, the "ash" in the original gelatin is much more effective as a clearing agent than the neutral salt added subsequently to the ash-free material.

The electrical conductivity of gelatin solutions.

The electrical conductivity of these solutions proves a useful criterion of the degree of purity of the gelatin. Compare for example the following:

	Conductivity of a 1 % solution at 25° Reciprocal ohms
A high grade commercial gelatin. (Coignet's Gold Label)	259×10^{-6}
The same after electro-dialysis	$20{\cdot}5 \times 10^{-6}$
After further purification by "recrystallisation" and precipitation in an electric field	$9{\cdot}5 \times 10^{-6}$

These conductivities refer to 1 % solutions, calculated on the weight of air-dried material, made up at 35°, and measured at 25° after about an hour in a thermostat at the latter temperature. The solutions of the purified gelatin, when kept at 25°, alter slowly in conductivity over the first 24 hours and thereafter remain constant. Thus the solution quoted above, which had a specific conductivity of $9{\cdot}5 \times 10^{-6}$ after an hour at 25°, had increased in conductivity to a value $12{\cdot}6 \times 10^{-6}$ after 24 hours. This increase in conductivity appears to run parallel with the slow flocculation of the hydrated gelatin.

The conductivity of a solution kept at 35° does not change appreciably with the time.

The specific conductivity of solutions of the highly purified gelatin is not proportional to the concentration. It varies as indicated by the following results:

Concentration of solution	Specific conductivity at 25°
0·5 %	$8{\cdot}15 \times 10^{-6}$
1·0	$9{\cdot}5 \times 10^{-6}$
2·0	$11{\cdot}9 \times 10^{-6}$

Note. The specific conductivity of the water used for making up the solutions in all the conductivity determinations was between $1{\cdot}0 \times 10^{-6}$ and $1{\cdot}5 \times 10^{-6}$.

Nelson Dale's Photographic Gelatin (a skin gelatin) gave similar results. Thus:

	Specific conductivity 1 % solution at 25°
Immediately after electro-dialysis	$15{\cdot}7 \times 10^{-6}$
After further purification	$11{\cdot}4 \times 10^{-6}$

The effect of heating the solution to 100° for a time, on the conductivity measured at 23°, is shown by the following results:

Initial specific conductivity of 1 % solution ...	$8{\cdot}1 \times 10^{-6}$
After 3 hours at 100°	$18{\cdot}2 \times 10^{-6}$
After 7 hours at 100°	$24{\cdot}2 \times 10^{-6}$

The increase in conductivity due to the degradation products on heating the solution at 100° is very marked.

France and Moran [1924] give the following results for the specific conductivity of solutions of a purified gelatin:

Concentration	Specific conductivity at 25°
1 %	$40{\cdot}09 \times 10^{-6}$
2 %	$67{\cdot}10 \times 10^{-6}$
(Water	$1{\cdot}0 \times 10^{-6}$)

This gelatin had been purified by the method described by Ada M. Field [1921]. This is the method due originally to Loeb.

The optical rotatory power of gelatin sols and gels.

The so-called "muta-rotation" of gelatin has been suggested by C. R. Smith [1919, 1920] as a criterion of the jelly strength, and therefore, presumably, of the freedom of the sample from breakdown products. The "muta-rotation" may be expressed as the ratio

$$\frac{\text{Equilibrium value of specific rotation at 15°}}{\text{Equilibrium value of specific rotation at 35°}}$$

For this ratio in a good sample of gelatin Smith gives the value 2·21. Poorer gelatins give lower values. Since, however, all samples of gelatin give very nearly the same rotation at 35°, the specific rotation at 15° may be used as the criterion of jelly strength of the sample.

Below are tabulated some values obtained for the purified Coignet Gold Label gelatin. All the measurements are made on a 5 % gel or sol. They are all equilibrium values, that is, the sol or gel was kept at the temperature mentioned until a constant value for the rotation was obtained.

Specific rotation at 35° in 0·1 *N* KCl	...	...	− 146°
,, ,, 15° in 0·1 *N* KCl	...	...	− 313°
,, ,, 15° in 1·0 *N* NaCl	...	...	− 313°
,, ,, 15° in 1·0 *N* sodium salicylate			− 144°

The neutral salt was introduced because otherwise the gel is rather opaque and it is difficult to get accurate readings. With salts such as potassium chloride or sodium chloride there is no change in rotation over large changes in concentration of the salt.

It is interesting to observe that in the presence of a salt which liquefies gelatin, such as sodium salicylate, there is practically no change in rotation on cooling from 35° to 15°. If the salt is removed again by dialysis the solution forms a gel at 15°, and the rotation at that temperature reverts to its former value.

The effect of heating for some time at 100° is shown by the following results:

	Specific rotation at 15°	Specific rotation at 35°
Initially	− 345°	− 142°
After 4 hours at 100°	− 255°	− 144°
After 14 hours at 100°	− 209°	− 145°

Heating at 100°, therefore, has practically no effect on the rotation at 35°, but, on the contrary, a marked effect on the equilibrium value at 15°.

It seems to the present writer to be misleading to refer to the change of rotation on cooling a gelatin sol as muta-rotation and to attribute it to a chemical change [cf. Smith, 1919, 1920]. The change in rotation is associated rather with the physical change of sol to gel, and is indeed, as Smith has shown, a convenient way of following that change quantitatively.

Viscosity.

It is well known that, in determining the viscosity of a gelatin solution, the pre-treatment of the solution is of the first importance. For a comparison of viscosities of different gelatins exactly the same method must be followed in preparing the solutions of the different samples. Moreover, since subjection of the solution to a high temperature even for a short time produces an irreversible decrease in viscosity, it is desirable that in making up the solution as low a temperature should be used as is convenient. Another difficulty, which may be mentioned here, arises when attempts are made to measure viscosities of gelatin solutions at temperatures at which they possess appreciable rigidity. Measurements determined in a simple Ostwald, or similar type, viscometer are unreliable with solutions which do not obey Poiseuille's law [cf. Bogue, 1922]. This difficulty, however, does not arise if the determinations are carried out at 35°, or above, with solutions which have been given time for the transition gel → sol to be completed.

A study of the variation of the viscosities of sols of the purified gelatin with time was made at various temperatures from 35° to 40°. In all the experiments the results of which are quoted below, 5 g. of the air-dried material, which contained 22·0 % moisture, were made up to 100 cc. The gelatin was weighed out into a graduated flask, covered with water and allowed to soak overnight at room temperature. Water was then added up to the mark, and the flask was placed in a thermostat at the temperature at which the viscosity was to be measured. As soon as the gelatin had completely dissolved, the sol was transferred to the viscometer and observations of the time of flow were made at suitable intervals. A simple Ostwald viscometer was used. It will be noted that in this procedure the temperature is never raised above that at which the viscosities are measured.

The results, when plotted, gave always the same type of curve (Fig. 1). The viscosity at first falls off rapidly with the time, but after one or two hours the rate of fall becomes very much less. This fall in viscosity, however, is continuous. In no case was a constant viscosity reached, though some of the observations at 35° were carried over periods of more than 100 hours.

If the sol is removed from the thermostat, allowed to cool for some time, and then returned to the higher temperature, the variations in viscosity which occur are illustrated in Figs. 2 *a* and 2 *b*. If the cooling period is short, the viscosity-time curve continues at practically the same point that it had

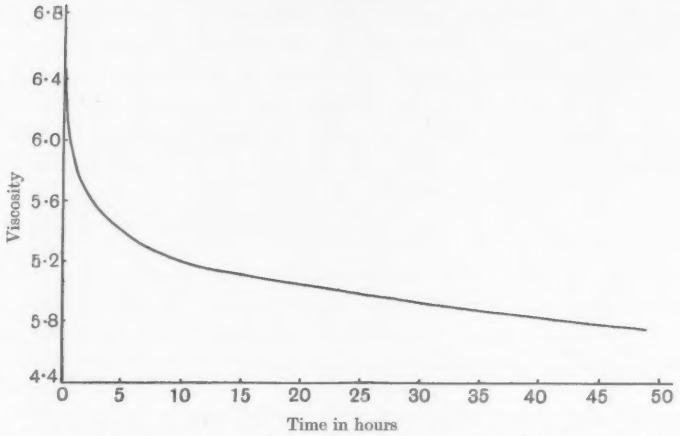

Fig. 1. Variation of viscosity with time. 5 % purified Nelson Dale Photographic Gelatin at 35°. $p_H = 4{\cdot}9$.

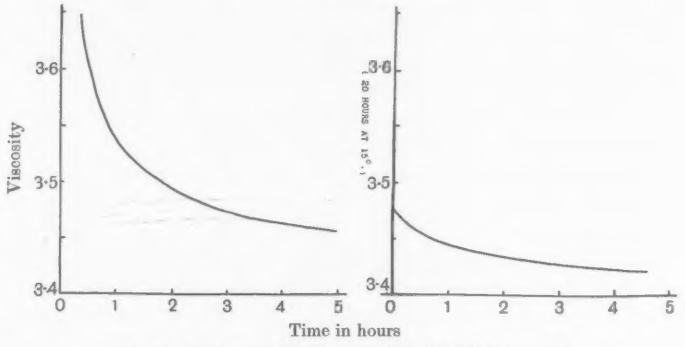

Fig. 2*a*. 5 % purified Coignet Gold Label Gelatin at 38°.

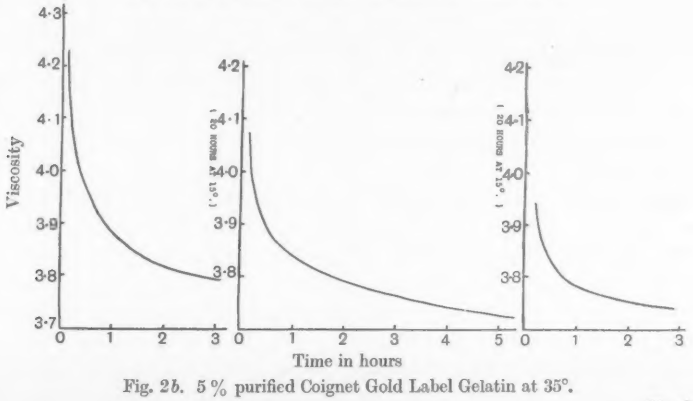

Fig. 2*b*. 5 % purified Coignet Gold Label Gelatin at 35°.

reached before cooling. With a longer period at the lower temperature a certain amount of recovery is observed. The recovery is more marked the lower the temperature at which the viscosities are measured (cf. Figs. 2 *a* and 2 *b*).

The initial fall in viscosity is due principally to the time taken by the transformation of gel to sol.

The results of these experiments show that even at 35° there is a slow *irreversible* decrease in viscosity of sols of highly purified gelatin.

The hydrogen-ion concentrations of the sols of the purified gelatin lay between p_H 4·90 and 4·95 at 35°. They did not vary appreciably with time.

It is evident that the initial viscosity as measured by the procedure outlined above is unsuitable as a basis for the comparison of the viscosities of different gelatins. Its rapid variation with time makes it dependent on the time taken for complete solution of the gelatin, which varies from sample to sample even of the same preparation. Moreover, as previously mentioned, reliable results cannot be attained by the use of an Ostwald viscometer until the transformation gel → sol is complete. The viscosities measured at a definite arbitrary length of time after immersion in the thermostat might be used. The time would have to be long enough for the rate of decrease of viscosity to have become very small; this would require at least 4 or 5 hours, which would be inconvenient.

If the sol is heated for a time to a higher temperature before being placed in the thermostat at 35° the initial rapid fall of viscosity is eliminated. Indeed, a 5 % sol which has been made up at 60°, and kept there for some time, exhibits a *rise* of viscosity with time on being brought to 35°. This rise, however, is only temporary. After a few hours the viscosity reaches a maximum and thereafter falls. By suitably adjusting the temperature at which the solution is made up and the time it is kept at that temperature, it is possible to keep the viscosity at 35° appreciably constant over a period of 3 or 4 hours. The period at the higher temperature has eliminated the initial steep portion of the curve mentioned above. When the solution is suddenly cooled to 35° there follows a slow rise in viscosity due to the time required by the sol to readjust itself to the new conditions, the "hysteresis" effect. This, superposed on the natural slow fall in viscosity at 35°, may result in an apparently constant viscosity. That the constancy is only apparent is shown by continuing the observations over a sufficiently long time; eventually the slow fall of viscosity reasserts itself.

The so-called "transition point," i.e. the temperature at which the viscosity does not change with time, observed by Davis and Oakes [1922] does not therefore possess the special significance they, and others [cf. Bogue, 1922], seem to attach to it. It is a function of the previous treatment of the sol. Davis and Oakes [1921] make up their solutions at 75°, which accounts for their finding a "transition point" about 38°. They give 38·03°.

If all that is required is a *comparison* of the viscosities of solutions of different

gelatins at 35°, the most convenient procedure is to make up the solutions at such a temperature and under such conditions that on cooling to 35° the viscosity remains appreciably constant for 2 or 3 hours. In obtaining the results quoted below, the solutions were made up at 50°. They were allowed to remain in the thermostat at 50° for one hour, and were then at once transferred to the thermostat at 35°. The subsequent variation of viscosity with time is illustrated by the typical curve shown in Fig. 3. The results are the average of readings taken repeatedly, especially during the first few hours.

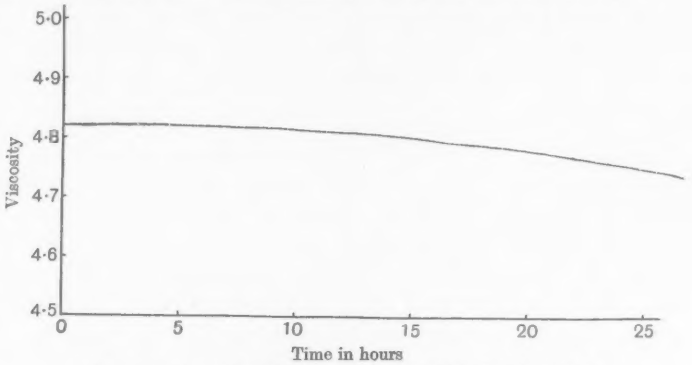

Fig. 3. 5 % purified Nelson Dale Photographic Gelatin at 35°, after heating at 50° for one hour.

RESULTS WITH NELSON DALE PHOTOGRAPHIC GELATIN.

Viscosities of 5 % solutions at 35°, relative to that of water at the same temperature.

	Viscosity	p_H
Before purification	5·036	6·3
After „	4·822	4·9

The apparent decrease in viscosity on purification is more than accounted for by the change in H-ion concentration. Addition of acid or alkali to the purified gelatin at once raises its viscosity. The variation of viscosity with p_H when the purified gelatin is dissolved in dilute alkali solutions, is shown in the following table. The H-ion concentrations were measured electrometrically at the same temperature at which the viscosities were determined (35°).

Purified gelatin dissolved	Viscosity	p_H
(1) In water	4·822	4·9
(2) In 0·002 *N* ammonia	4·933	5·0
(3) In 0·006 *N* NaOH	5·399	5·7
(4) In 0·017 *N* NaOH	7·074	10·2

In the last-mentioned case, i.e. at p_H 10·2, the viscosity at 35° did not show the initial constant period, but decreased continuously with time. That given is the initial viscosity.

It is evident that, if the comparison is made *at the same H-ion concentration*, the method of purification used results in an increase of viscosity.

Conclusions.

(1) The amount of nitrogen remaining in solution after the electrodialysis of a gelatin forms a good criterion of its degree of separation from degradation products—this amount in the particular sample investigated lay between 1 and 2 mg. per 100 cc. when the lower pole in the dialysis apparatus was the anode and the proper movement of the gelatin in the electric field was in the same direction as gravity.

(2) The previous determination of the solubility (Knaggs, Manning and Schryver) of 0·056 part of gelatin in 100 cc. of water was too high, as complete equilibrium between the phases in the experiments described had not been attained, and the gelatin was probably not completely freed from the last traces of electrolytes.

(3) If a purified gelatin has any true solubility in water (i.e. if it can be "molecularly dispersed") this does not exceed 10 parts in 100,000.

(4) A gelatin is not a protein *sui generis*, but has nearly all the characteristic properties of a globulin. It is insoluble in water, but soluble (or dispersible) in very weak solutions of acids and alkalis. Very much stronger solutions of neutral salts such as potassium chloride are necessary to produce corresponding effects. It differs from most other globulins in its higher state of hydration, to which may be ascribed the great capacity for forming gels. The other globulins can also be made to form gels when they can be dispersed in a relatively small amount of dispersion medium. Thus, for example, edestin, a typical plant globulin, will yield a jelly when obtained in high concentration in a solution of sodium salicylate.

(5) Gelatin solutions show an irreversible diminution in viscosity when kept, even at 35°. The conditions to be observed for obtaining correct determinations with an Ostwald viscometer are discussed.

The above work was done under the direction of Professor S. B. Schryver.

References.

Bogue (1922). *J. Amer. Chem. Soc.* **44**, 1313.
Davis and Oakes (1921). *J. Amer. Chem. Soc.* **43**, 1526.
—— —— (1922). *J. Amer. Chem. Soc.* **44**, 464.
Field (1921). *J. Amer. Chem. Soc* **43**, 668
France and Moran (1924). *J. Amer. Chem. Soc.* **46**, 27.
Knaggs, Manning and Schryver (1923). *Biochem. J.* **17**, 473.
Knaggs and Schryver (1924). *Biochem. J.* **18**, 1079.
Smith, C. R. (1919). *J. Amer. Chem. Soc.* **41**, 135.
—— (1920). *J. Ind. Eng. Chem.* **12**, 878.

CXLI. INVESTIGATIONS ON GELATIN. PART VI. THE INFLUENCE OF THE TREATMENT OF THE PRECURSOR ON THE CHARACTER OF THE GELATIN.

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Report to the Adhesives Research Committee of the Department of Scientific and Industrial Research.

(*Received July 30th, 1924.*)

The experiments described in this communication had the following objects: A. The determination of the character of the products separated from gelatin during flocculation in an electrical field. B. To determine whether gelatins after repeated purifications by flocculation possessed the same properties when obtained from different precursors, or different treatments of the same precursor, in other words, to ascertain whether gelatin could be obtained as a definite chemical entity.

A. Chemical Characters of the Products separated during the Flocculation of Gelatin in an Electric Field.

General Method of Experiment.

The gelatin prepared from purified ossein was freed from electrolytes by washing with frequent changes of distilled water for 4 days, and finally the last traces of soluble salts were removed by electrolysing a 20 % gel. The Hausmann numbers of the fractions obtained were determined.

5 litres of a 2 % solution of this purified gelatin were prepared, and flocculated in the apparatus described. When the number of milligrams of nitrogen per 100 cc. of the supernatant liquid reached a constant value, which was after the current had passed through the solution for 5 days, the clear supernatant liquid was syphoned off and filtered to remove any particles of undissolved gelatin. This liquid was concentrated under reduced pressure at 40°, and then hydrolysed with 20 % hydrochloric acid.

The flocculated gelatin was rendered free from the supernatant liquid by repeatedly washing by decantation with distilled water. A portion of the flocculated and washed gel was hydrolysed with 20 % hydrochloric acid, the remainder was made up to a 2 % solution by adding the calculated quantity of water and warming to 37°. This solution was reflocculated by the electric current.

The liquid in the cathode compartment which contained the part which diffuses through the membrane, was reduced to small bulk by evaporating under reduced pressure at 40°, and hydrolysed with 20 % hydrochloric acid.

The Hausmann numbers of the three fractions were determined.

The process of flocculation was repeated several times, and each time the Hausmann numbers of the three fractions were found.

The above experiment was carried out using a gelatin prepared from ossein which had been treated for 60 days with 0·2 % NaOH and for 1 day with 0·5 % HCl. The ossein was thoroughly washed in running water to remove all traces of free acid before extraction of the gelatin. The experiment was repeated using gelatin extracted from ossein which had had a prolonged treatment with 0·2 % NaOH only, and in these experiments the supernatant liquid and liquid in the cathode compartment were acidified with dilute hydrochloric acid before evaporation to small bulk to retain in solution any ammonia or volatile nitrogen compounds. The following are the results of the first experiment:

Table I.

Exp. 1. Gelatin from ossein treated for 60 days with 0·2 % NaOH and for 1 day with 0·5 % HCl

	Amide N	Humin N	Diamino N	Mono-amino N	Non-amino N
A. Original gelatin	2·0	1·1	22·86	—	—
B*. Electrolyte-free gelatin	1·97	1·86	22·59	64·12	9·46
First flocculation of 2 % solution of B					
Supernatant liquid	1·90	2·12	20·94	—	—
Flocculated gelatin	2·01	2·01	21·38	60·49	11·94
Liquid which diffused to cathode	1·6†	1·57	15·95	—	—
Second flocculation					
Supernatant liquid	1·97	2·16	20·61	—	—
Gelatin flocculated twice	2·06	1·93	21·59	61·37	13·05
Liquid which diffused to cathode	1·54†	1·30	12·73	—	—
Third flocculation					
Supernatant liquid	2·63	2·83	19·71	—	—
Gelatin flocculated three times	1·96	1·99	21·68	57·49	16·88
Liquid which diffused to cathode	2·41†	1·73	13·72	—	—
Fourth flocculation					
Flocculated gelatin	2·06	2·16	21·61	56·03	18·14
Seventh flocculation					
Flocculated gelatin	1·86	2·42	21·68	55·99	18·05
Eighth flocculation					
Flocculated gelatin	1·91	2·33	21·76	55·30	18·7
Exp. 2. Gelatin from ossein treated with 0·2 % NaOH only for 60 days.					
C. Original gelatin	1·54	1·26	24·09	—	—
D‡. Electrolyte-free gelatin	1·78	1·31	26·27	60·40	10·24
First flocculation of 2 % solution of D					
Supernatant liquid	2·55	3·02	23·03	58·21	13·19
Flocculated gelatin	1·91	3·77	24·12	55·21	14·99
Liquid which diffused to cathode	3·89	3·28	21·02	38·8	33·01
Second flocculation					
Supernatant liquid	2·57	1·73	20·69	61·21	13·8
Flocculated gelatin	1·74	1·18	24·89	53·04	19·15
Liquid which diffused to cathode	10·08	2·60	12·76	43·28	31·28
Third flocculation					
Supernatant liquid	6·31	3·06	17·95	58·11	14·577
Flocculated gelatin	1·76	1·49	24·68	52·17	19·91
Liquid which diffused to cathode	9·35	7·79	8·83	31·66	42·37

* Electrolytes removed from A by electrolysis of 20 % gel.

† The percentage of amide nitrogen is low because the liquid which diffused to the cathode compartment was not acidified with hydrochloric acid before concentration under reduced pressure.

‡ C was washed with water and a 20 % gel electrolysed.

The deductions from Exps. 1 and 2 may be summarised as follows:

(*a*) *Supernatant liquid.* This is the more acidic fraction as it collects round the anode, and this is borne out by the percentage of diamino nitrogen being lower than that of the flocculated gelatin. The non-amino nitrogen number is also lower than that for the precipitated gelatin and remains fairly constant throughout the experiment.

The amide nitrogen values in Exp. 2 are much higher than the corresponding values in Exp. 1, as precautions were taken in the second experiment to retain any volatile nitrogen compounds before concentration under reduced pressure. These high values may be due to a slight oxidising effect of the anode.

(*b*) *Flocculated gelatin.* The diamino nitrogen number becomes constant after the first flocculation, but the percentage of non-amino nitrogen increases at each flocculation to a constant value. This observation is treated in greater detail in the next communication.

(*c*) *The liquid which diffuses through the membrane to the cathode.* This fraction has abnormally low values for the percentage of diamino and mono-amino nitrogen. This fraction is basic in character seeing that it is attracted to the negative pole, and it would therefore be expected that the percentage of diamino nitrogen would be greater than that of the other fractions. The results in Exps. 1 and 2 do not show this to be the case, the reason being that no precautions were taken to prevent secondary reactions due to the reducing action of the cathode. The high percentage of amide nitrogen in Exp. 2 would indicate that ammonia or its simple derivatives were among the products formed, and the high percentage of non-amino nitrogen that the diamino and mono-amino nitrogen groups underwent molecular rearrangement forming ring compounds such as pyrroles, pyridines and piperazines, etc.

(*d*) *The differences in the gelatins used in the above experiments.* The most striking result indicated by Exps. 1 and 2 is the difference in the Hausmann numbers of the gelatins which have been extracted from ossein treated with acid and alkali respectively. When the last treatment has been with acid a gelatin is obtained which yields a lower diamino nitrogen number than a gelatin extracted from an ossein the last treatment of which has been with an alkali. These results were obtained in spite of the fact that the ossein had been subjected to very thorough washing with water, whether the last treatment had been with an acid or an alkali.

B. Influence of the Treatment of the Precursor on the Character of the Gelatin.

The above experiments indicate that gelatins differ according to whether the precursors from which they have been extracted received a final treatment with acid or alkali. These experiments have been extended to other precursors and similar results have been obtained which are shown in Table II.

The horn-pith and Achilles tendon of the ox were used. They received a preliminary treatment similar to that described for ossein, in that they were extracted with 0·2 % NaOH for 60 days to remove organic nitrogen impurities, and then thoroughly washed in running water. The precursors were then treated as follows:

(*a*) with 0·2 % NaOH,

(*b*) with 0·5 % HCl

for 5 days. The excess of free acid or alkali was removed by washing and the gelatin, extracting at 80°, concentrated, dried in a current of air, washed with water and twice flocculated from a 1 % solution in an electric field, taking the precautions described on p. 1095.

Table II.

Precursor	Final treatment of precursor before washing and extraction	Amide N	Diamino N	Difference in Diamino N
Horn-pith	0·2 % NaOH	1·30	24·20	1·26
Horn-pith	0·5 % HCl	1·45	22·94	
Tendon*	0·2 % NaOH	3·02	29·50	3·50
Tendon*	0·5 % HCl	3·45	26·00	
Bone†	0·2 % NaOH	3·2	21·39	1·61
Bone†	0·5 % HCl	3·1	19·78	
Ossein	0·2 % NaOH	1·74	24·89	3·30
Ossein	0·5 % HCl	2·06	21·59	

* The tendons were cut into small pieces and treated with 0·2 % NaOH for 14 days. They were washed free from alkali and preserved in alcohol for several months when they were dried and treated for several weeks with 0·2 % NaOH. The Hausmann numbers of the tendon gelatin differ markedly from those of horn-pith or ossein gelatin. Whether this difference is due to the fact that the gelatins are not the same, or whether tendon gelatin is contaminated with other products which do not exist in horn-pith or ossein cannot be as yet definitely stated.

† The bone was treated with the calculated quantity of 0·5 % HCl to remove the carbonate, washed free from acid and the gelatin extracted at 95°. The acid-treated bone was treated with 0·2 % NaOH for 2 days, well washed, and the gelatin prepared from it. These samples of gelatin contain impurities such as chondroitinsulphuric acid, and they were not purified either by washing or flocculation, yet they show a difference in the percentage of diamino nitrogen.

In all the above samples of gelatin, there is a difference in the percentage of diamino nitrogen depending on the previous treatment of the precursor. A gelatin with the higher percentage of diamino nitrogen was always obtained from a precursor which had had a final treatment with alkali before being washed and extracted, the one with the lower value for diamino nitrogen being obtained from a precursor which had had acid treatment.

A probable explanation of this fact may be found in assuming that gelatin can exist in two tautomeric forms, keto and enol [compare Dakin, 1910, 1912, 1913; Dakin and Dudley, 1913; Robertson, 1920; Jordan Lloyd, 1920], and that ordinary gelatin is an equilibrium mixture of the two. The same modifications will exist in the precursors of gelatin, and the keto form will be changed into the enol form and *vice versa*, according to conditions, i.e. the reaction is reversible and will probably depend on the p_H.

Precursor of gelatin (keto form) $\rightleftarrows$ Precursor of gelatin (enol form).

The gelatins obtained from horn-pith, tendon or ossein, are markedly different in their physical properties. The above numbers indicate that they are different chemically.

A similar result was obtained from the hydrolysis products of the precursors. If the latter are rapidly hydrolysed after treatment with acid they yield a smaller amount of diamino acids than if hydrolysed after treatment with alkali. The difference in the diamino acids amounts to nearly the same as was obtained from the corresponding gelatins. The hydrolysis products of the precursors will be dealt with in a later communication.

Rate of Extraction.

In view of the above results it was of interest to ascertain whether differences could be observed in the rate of extraction of gelatin according to whether the final treatment of the precursor had been with acid or alkali. The following experiments were carried out and the method employed was that described by Manning and Schryver [1921].

Rate of Extraction of Gelatin from Horn-pith, at 100°.

The horn-pith was purified in the same way as the ossein by washing for several weeks with 0·2 % NaOH and was then treated as follows, and the rate of extraction followed for samples A, B, C and D.

A. Horn-pith treated with 0·5 % HCl for 2 days.

B. A was electrolysed for 10 days in the apparatus described for flocculating gelatin.

C. D was electrolysed for 10 days.

D. Horn-pith treated with 0·2 % NaOH.

The results are shown graphically in fig. 1.

In these experiments all the free acid or alkali was removed from the tissues A and D by a prolonged washing in running water and finally with distilled water.

Rate of Extraction of Gelatin from Ossein at 92°.

The ossein used was freed from all organic nitrogen impurities by prolonged treatment with 0·2 % NaOH. The following results were obtained (Table III):

Table III.

Ossein treated with caustic soda previous to extraction		Ossein treated with hydrochloric acid previous to extraction	
Time in hours	% extracted	Time in hours	% extracted
0·5	21·8	0·5	20·8
1·25	35·4	1·5	57·3
2·0	46·0	2·5	76·1
3·0	61·5	3·5	92·0
4·0	71·9	4·16	95·9
5·0	78·1	5·16	99·7
6·0	81·9	6·16	99·0
—	—	7·33	100*

* It was assumed in these calculations that all the gelatin was extracted after 7 hours. The percentages in both columns are based on this assumption.

It is seen that the gelatin is extracted more rapidly from the acid-treated precursor than from either the alkali-treated precursor or a precursor which had been submitted to electrodialysis. A similar result was obtained by following the rate of extraction of gelatin from finely powdered bone. If the bone is partially decalcified with 0·5 % HCl the rate of extraction is much quicker than if the same sample is treated with 0·2 % NaOH for 2 days after the acid treatment, for example, after 6 hours at 95°

43% of gelatin is extracted from acid-treated bone,
37% „ „ „ alkali-treated „

Another point worthy of notice is that a precursor which had been treated with acid, washed and dried, was more deeply coloured than the same precursor which had had alkali treatment.

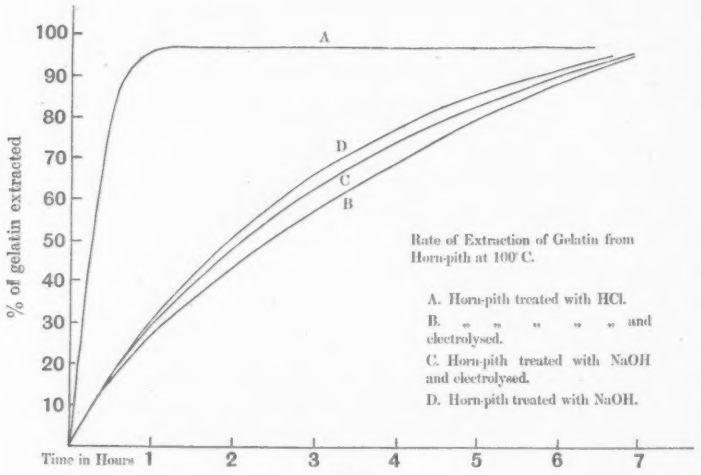

Fig. 1.

The gelatin extracted from an acid-treated precursor has always a deeper shade of colour than the gelatin from the alkali-treated precursor. Gelatin from alkali-treated horn-pith is almost colourless. It seems, therefore, probable that the acid-treated precursor and the alkali-treated precursor are not chemically identical.

Conclusions.

(1) A highly purified gelatin on flocculation from 2 % solution in an electric field separates into three fractions. The supernatant liquid round the anode contains a fraction with a low diamino nitrogen value; the portion which diffuses through the membrane to the cathode is reduced by the pole into simple products. The amount of these two fractions is relatively small compared with the amount of gelatin which is flocculated.

(2) Repeated flocculation of the gelatin from 1 % solution in an electric field does not alter the percentage of diamino nitrogen, but the non-amino nitrogen increases at each flocculation to a maximum of 20 (approx.).

(3) Gelatins differ in their Hausmann numbers and this depends on the previous treatment of the precursors before extracting the gelatin. Gelatin extracted from a precursor which has had acid treatment before extraction has a lower value for the diamino nitrogen than the gelatin extracted from the precursor which has had alkali treatment. These differences amount to between 1·5–3·5 %. A possible explanation of these facts is that the precursor undergoes the keto-enol change.

(4) The rate of extraction of gelatin from an acid-treated precursor is much greater than from the same precursor which has had alkali treatment, or has been submitted to electrodialysis.

(5) There is no reason for regarding "gelatin," even after submission to elaborate methods of purification as a definite chemical entity.

REFERENCES.

Dakin (1910). *J. Amer. Chem. Soc.* **44**, 48.
—— (1912). *J. Biol. Chem.* **13**, 357.
—— (1913). *J. Biol. Chem.* **15**, 271.
Dakin and Dudley (1913). *J. Biol. Chem.* **15**, 271.
Jordan Lloyd (1920). *Biochem. J.* **14**, 147.
Manning and Schryver (1921). *Biochem. J.* **15**, 523.
Robertson (1920). The Physical Chemistry of the Proteins, 24.

CXLII. INVESTIGATIONS ON GELATIN. PART VII. THE NON-AMINO NITROGEN OF GELATIN.

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Report to the Adhesives Research Committee of the Department of Scientific and Industrial Research.

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In the purification of gelatin by flocculation in an electric field, the endeavour was made to separate gelatin from the products of its thermal degradation, produced in the course of its preparation from its precursors. To amplify this work, a series of experiments was undertaken with the object of following in some detail the process of separation of the products of thermal degradation from a gelatin which had been highly purified by repeated flocculation and subsequently heated with water. During the course of these experiments a certain chemical change in the protein molecule was noted which does not appear to have been previously recorded. If a freshly prepared purified gelatin is submitted to rapid hydrolysis by acids, and the Hausmann numbers are determined, it will be generally found that the non-amino nitrogen is relatively low. If the same gelatin be heated, and the degradation products afterwards separated by the method of flocculation in an electric field, a product will be obtained, of which (after rapid hydrolysis by acids) the Hausmann numbers will be found to differ markedly from those of the original gelatin in only one factor. It will be found that there has taken place a marked increase in the non-amino nitrogen. This phenomenon may be most plausibly explained by assuming that the addition of hot water produces a molecular re-arrangement in the protein molecule (or molecules) with the formation of closed rings of the nature of proline or ketopiperazines in which an amino-group takes part, which are resistant to hydrolysis with acids and do not yield nitrogen on treatment with nitrous acid as did the amino groups which were responsible for this ring formation.

These observations also offer a partial explanation of another fact. Although gelatin, on heating with water, rapidly undergoes an irreversible change, yielding simpler products which rapidly diffuse, no increase in the amino nitrogen could be detected by the methods of Sörensen or Van Slyke in the degraded gelatin, as would be expected, had any scission of peptide linkages taken place. The formation of stable ring compounds from amino groups would effectively mask an increase in such groups due to this scission.

It was also found that an increase in the non-amino nitrogen of the hydrolysis products took place when gelatin was repeatedly flocculated, or when it was dissolved in cold solutions of acids and alkalis.

Experimental.

Flocculation of a purified gelatin which had been subjected to the action of heat.

A 2 % solution of a bone gelatin which had been purified by four flocculations was heated in a quartz vessel under a reflux for 5 hours. The solution was then flocculated in an apparatus similar to that already described [Knaggs and Schryver, 1924]. A control experiment was carried out in an exactly similar apparatus, and as nearly as possible under the same conditions with a 2 % solution of the same gelatin which had not been subjected to heat. 500 cc. were introduced into the flocculating tube in each case, and 500 cc. of water into the beaker which contained the mercury cathode. At intervals 25 cc. were pipetted out of both the supernatant fluid and the liquid round the cathode (which contained substances which had passed through the collodion membrane), from both sets of apparatus, and the nitrogen was determined in these samples. The results are recorded in the following table:

Table I.

	Heated gelatin solution		Unheated gelatin solution	
Time in hrs.	Supernatant fluid mg. N/100 cc.	Cathode liquid mg. N/100 cc.	Supernatant fluid mg. N/100 cc.	Cathode liquid mg. N/100 cc.
0	230·6	—	230·6	—
28	51·56	9·92	60·8	1·13
53	34·28	10·9	22·36	1·56
100	25·16	14·1	10·2	1·41
197	22·36	18·7	5·5	1·42
250	21·2	19·2	3·7	1·50
315	20·94	20·68	3·5	1·59

It will be noticed that the nitrogen of the supernatant fluid from the heated sample becomes constant after about 250 hours, and amounts to 21 mg./100 cc. which is in great contrast to the corresponding figure when constancy has been obtained of the unheated sample, which is only 3·5 mg./100 cc. The following are the Hausmann numbers of the various samples:

Table II.

	Amide N	Humin N	Diamino N	Mono-amino N	Non-amino N
A. Original gelatin purified by 2 flocculations	2·06	1·93	21·59	61·37	12·32
B. Original gelatin, purified by 2 flocculations, of which a 2 % solution had been heated for 5 hours	2·08	3·81	22·64	54·2	17·27
C. Gelatin produced from B by flocculation	1·78	1·63	22·2	56·1	18·29
D. C re-flocculated	1·85	2·98	21·42	56·60	22·15

It will be noticed from the table above that, with the exception of the non-amino nitrogen, the variations are small and irregular, and these are due to

the somewhat limited accuracy of the experimental methods. There is, however, a marked change in the non-amino nitrogen.

A similar experiment was carried out with another sample of gelatin which had been flocculated seven times. In this case the non-amino nitrogen of the unheated gelatin was high to start with and was 18·05 %[1]. When it was heated for 5 hours in a 2 % solution, and the solution was then placed in an electric field, a gelatin was flocculated which gave a non-amino nitrogen of 20·87 %. Again a distinct increase produced by heating.

The dependence of the non-amino nitrogen on the length of the treatment of the gelatin with hot water. In the following experiment a sample of Coignet's gold label gelatin was used, which had been freed from electrolytes by electrolysis, and had been subsequently "recrystallised" once [Knaggs, Manning and Schryver, 1923]. This yielded on analysis the following numbers:

Table III.

	Amino N by						
	Van Slyke's method	Sörensen's method	Amide N	Humin N	Diamino N	Mono-amino N	Non-amino N
Coignet's ash-free gold label gelatin, "recrystallised" once	5·1	5·1	1·02	0·74	22·34	64·19	11·71
	Determined before hydrolysis		Determined after hydrolysis				

A 5 % solution of this gelatin was heated in a quartz vessel in an oil-bath at 105° under a reflux. A sample was removed at intervals, hydrolysed and the Hausmann numbers determined. The humin and diamino N showed the usual slight irregular variations, but the non-amino N continuously increased till it became constant. The results are indicated in the accompanying curve (Fig. 1).

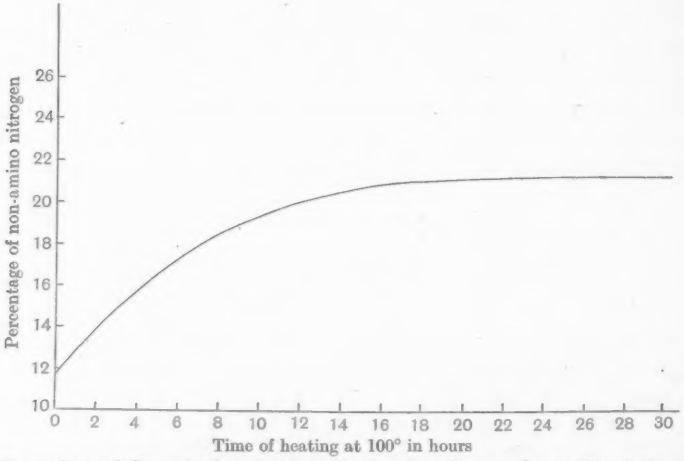

Fig. 1. Rate of change in the percentage of non-amino nitrogen when a 5 % solution of ash-free Coignet's gold label gelatin is heated at 100° C.

[1] For explanation of this high number see remarks in introduction and in sequel.

The solution of the heated gelatin would no longer flocculate in an electric field and the liquid round the cathode containing the portion readily dialysable gave a high value for the diamino nitrogen [cf. Knaggs, Manning and Schryver, 1923, p. 4]. When the solution was tested both by the Van Slyke and Sörensen methods, it showed the same percentage of amino N (5·1 %) as the unheated solution. This same figure was obtained even when solution had been heated 100 hours. The percentage of amide nitrogen remained constant at 1·1 % after 40 hours of heating at 100°.

Effect of flocculation on the non-amino N.

As noticed in the introduction, the non-amino nitrogen increases with successive flocculations. The following numbers taken from another paper will illustrate this point. Only these figures are quoted as there are only slight irregular variations in the other Hausmann numbers.

Table IV.

Gelatin	Non-amino N
Original gelatin (ash-free)	9·34
The same, once flocculated	12·32
The same, twice flocculated	13·05
The same, after 7 flocculations	18·05
The same, after 2 flocculations and heating the flocculated gel in 2 % solution 5 hours	17·27

The action of cold dilute acids and alkalis on the non-amino N.

A gelatin prepared from ossein, which had been rendered ash-free by electrolysis and once flocculated, was used for the experiments. A 3 % solution of this was treated with an equal volume of 2 *N* hydrochloric acid; and the mixture was allowed to stand for 48 hours. After this interval all the gelatin had gone into solution. The Hausmann numbers were then determined, after hydrolysis. A similar experiment was carried out by diluting the 3 % gelatin solution with an equal volume of 2 *N* sodium hydroxide. In this case the mixture was allowed to stand for 24 hours only before hydrolysis. The Hausmann numbers of the original and the acid- and alkali-treated gelatins are shown in the following table.

The gelatin which had been treated with acid formed a gel when the acid was removed. This was not the case with gelatin which had been treated with alkali.

Table V.

	Amide N	Humin N	Diamino N	Mono-amino N	Non-amino N
A. Gelatin electrically flocculated once	2·06	3·53	21·60	60·49	12·32
A was treated with *N* HCl for 48 hours, i.e. until all gelatin was in solution, and immediately hydrolysed with 20 % HCl	2·25	1·23	20·91	55·12	20·49
A was treated with *N* NaOH for 24 hours, neutralised, and quickly hydrolysed	1·93	1·94	20·94	52·30	22·89

Conclusions.

When a gelatin purified by flocculation is heated with water and then hydrolysed, a marked increase in the nitrogen of the hydrolysis products which will not react with nitrous acid to give gaseous nitrogen is observed. This indicates in all probability a molecular re-arrangement in the molecule with the formation of stable ring compounds. A similar result is produced by repeated flocculation in an electric field and also when gelatin is treated with cold dilute acids and alkalis. The results indicate furthermore the variations which can exist in different samples of gelatin derived from the same precursors. It is not yet possible to consider gelatin as a definite chemical entity even after submission to purification by the methods which have been discussed in earlier papers.

References.

Knaggs, Manning and Schryver (1923). *Biochem. J.* **17**, 473.
Knaggs and Schryver (1924). *Biochem. J.* **18**, 1079.

CXLIII. INVESTIGATIONS ON GELATIN. PART VIII. THE OSMOTIC PRESSURE OF GELATIN IN SOLUTIONS OF SODIUM SALICYLATE.

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Report to the Adhesives Research Committee of the Department of Scientific and Industrial Research.

(*Received July 30th, 1924.*)

The present investigation was undertaken under the direction of Professor S. B. Schryver with a view to ascertaining the osmotic pressure of gelatin. Previous workers on this problem have not obtained their gelatin absolutely free from ash. Although the view is now commonly held that a low ash has no direct influence on the osmotic pressure it was thought desirable, since gelatin of exceptional purity has recently been prepared in this department [1923] to study the osmotic pressure of this preparation.

Since the osmotic pressure of gelatin is extremely low it is necessary to employ a fairly strong solution in order to obtain a pressure which can be easily and accurately measured with a very simple osmometer such as has been used in this investigation. If water be used as the solvent it is not possible to work at ordinary room temperature owing to the formation of a gel. It was therefore decided to employ as the solvent of the gelatin a solution of sodium salicylate, which, whilst having no chemical action upon the colloid, prevents gel formation. The liquefaction of gelatin by certain salts has been proved to be entirely reversible by Briggs and Hieben [1920] and the present investigator finds this to be the case with sodium salicylate. This reversibility proves that the salt in question has no chemical action upon the gelatin. The presence of this salt also serves to keep the solution sterile. A strong solution of sodium salicylate has therefore been used as the solvent of the gelatin in all the experiments herein described.

The Apparatus used.

The osmometer is very simple in form. The membrane was prepared from a 10 % solution of a purified collodion ("Necolloidine") dissolved in equal parts of alcohol and ether. To prepare the membrane a test-tube is dipped into the solution and immediately inverted in a conical flask (to prevent evaporation of the solvent) and allowed to drain for a minute or so. It is then dried for about 15 seconds in air before plunging into water[1]. The collodion sac is fitted with a straight rubber stopper through which is

[1] The writer is indebted to Dr Brown for his kind advice on the preparation of the osmometer.

passed a length of glass tubing. This acts as the manometer. The osmometer is filled through the manometer tube by attaching to it a small funnel containing the solution of gelatin, alternately expelling air from the sac and allowing some of the solution to take its place until it is filled to a convenient distance up the tube. It is supported in a vessel containing the solution used as solvent of the gelatin. A piece of sheet rubber, through which the manometer tube passes, is fixed (by means of a rubber band) over the top of the outer vessel to prevent concentration of the solution by evaporation. Three samples of gelatin prepared in this laboratory by Mr A. B. Manning have been studied. They are:

1. Coignet's Gold Label gelatin dialysed at the iso-electric point. Ash 0·054 %.

2. The same gelatin purified electrically [Knaggs, Manning and Schryver, 1923]. This sample was ash-free.

3. The same gelatin purified electrically and "recrystallised" [see Knaggs, Manning and Schryver, 1923]. Also free from ash.

The first series of experiments was carried out with a 5 % solution of the gelatin in *M* solution of sodium salicylate. Since this was found to be somewhat on the alkaline side of the iso-electric point—the p_H of the solution at the termination of the experiment being 6·3—two other series were prepared by adding salicylic acid to the solution of sodium salicylate until a solution with p_H 5·0 was obtained for one series and p_H 4·6 for a third series.

In series 1, when the osmometer was set up the p_H of the solution of gelatin was 5·4, that of the *M* sodium salicylate outside the membrane was 7·0. At the termination of the experiment the p_H was again determined in both the inner and outer solutions and was found to be in each case from 6·2 to 6·4. In series 2 and 3 where the initial p_H was the same on both sides of the membrane no subsequent change was observed. A Walpole electrode was used for the measurement of the hydrogen ion concentration, and, since it was found impossible to obtain a constant E.M.F. reading with the strong solution under investigation it was diluted with water to about 10 times its volume for the hydrogen ion determination in each case.

To prepare the solution about 5 g. of gelatin were added to 100 cc. of the solvent and warmed to 37° in an incubator, shaking occasionally until dissolved. Osmometers were filled in the manner described, the solution in the outer vessel being in each series the same as that used for dissolving the gelatin, and the height of the column in the manometer tube was noted. It was observed that in each case the pressure of the solution within the osmometer rose fairly rapidly, reaching a maximum in most cases within 6 or 7 days. It remains constant for a few days, then falls very gradually (see Fig. 1). This gradual fall of pressure continues even after six months' standing. Biltz, Bugge and Mehler [1916] observed a similar phenomenon working with a solution of gelatin in water, but they used a very dilute solution (0·1 to 0·25 %) and their observations extended over a relatively short period of time. The curve given

herein is representative of the change of pressure which occurs within the osmometer. A number of osmometers were dismantled when the pressure had remained constant for a few days, i.e. when it was at a maximum, the strength of the gelatin solution was determined by an estimation of the nitrogen content and the aggregate weight calculated by the usual method.

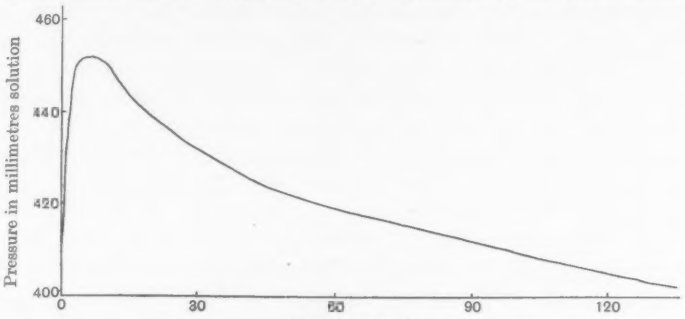

Fig. 1

The results are given in the following table:

Series 1. p_H 6·3.

Sample 1. Ash 0·054 %

Nitrogen in 5 cc. mg.	% gelatin in solution	Pressure in mm. solution	Temp. ° C.	Aggregate weight
30	3·33	451	14	16,620
26·8	2·98	402	14	16,640
			Average	16,630±1 %
		Sample 2. Ash-free		
32·5	3·61	482	13	16,750
32·5	3·61	510	16	16,020
31·5	3·5	494	14	15,970
			Average	16,246±3 %
		Sample 3. Ash-free		
25·15	2·79	382	13	16,380
27·8	3·09	420	17	16,700
			Average	16,540±1 %

Series 2. p_H 5·0.

Sample 3. Ash-free

27·3	3·04	419	14	16,300
26·56	2·95	402	15	16,550
30·5	3·39	465	16	16,480
30·0	3·33	465	18	16,490
29·0	2·96	480	22	15,710
			Average	16,300±4 %

Series 3. p_H 4·6.

Sample 2. Ash-free

30	3·33	405	15	18,540
32·8	3·63	472	15	17,380
29	3·22	394	17	18,570
30	3·33	436	19	17,480
			Average	17,990±4 %

Estimations of nitrogen were also made on the solution in the outer vessel at the conclusion of the experiment and the amount found was negligible, being in each case not more than 2 mg. nitrogen in 120 to 130 cc. solution.

For calculating the aggregate weight of the samples of gelatin used in this investigation it has been assumed that the maximum pressure obtained within the osmometer represents the osmotic pressure of the solution. The aggregate weights so calculated for the different samples of gelatin agree very closely, but from this investigation it appears that it is not possible to obtain a condition of equilibrium in a solution of gelatin, since it does not exhibit a constant osmotic pressure even after prolonged standing. The osmometer represented in the curve given herein was allowed to remain for 6 months undisturbed. It was found that the outer solution—which was 116 cc. in volume—contained only 2 mg. N. The solution removed from within the osmometer measured 19 cc. and contained 115 mg. N. The fall in pressure observed over this period of time may possibly be attributed to an increase in the average size of the molecular aggregate of the gelatin, the maximum pressure (which was attained after 6 days) being due to an average aggregate weight of 16,600 which has increased during the period of observation to 19,000. It might also be due to a gradual distension of the membrane, although Biltz and his co-workers [1916] obtained similar results using a rigid osmometer.

From the results recorded in the above tables it will be seen that there is no difference in the osmotic behaviour of the three samples of gelatin investigated, determinations made of each sample at the same hydrogen ion concentration (series 1, p_H 6·3) showing no difference in the aggregate weight as determined by the osmotic pressure. The presence of a very low ash in gelatin which has been dialysed at the iso-electric point therefore appears to have no influence on the osmotic pressure. The work of Loeb [1922] shows that the osmotic pressure is dependent on the p_H of the solution. The osmotic pressure is a minimum at the iso-electric point. It is a generally accepted theory that the increase in pressure which occurs with an increase in p_H is due to the formation of a salt of gelatin which ionises. The presence of another salt —sodium salicylate—increasing the concentration of the sodium ions will suppress such ionisation, so giving rise to a smaller increase in pressure as the p_H of the solution increases beyond the iso-electric point than would be the case in the absence of such a salt. The results of this investigation seem to confirm this. The increase in osmotic pressure is evidently small. Although in these results the pressure at p_H 5·0 appears to be the same as that at 6·3 whilst that at 4·6 is somewhat lower, it is probable that the pressures herein recorded at p_H 5·0 are all a little higher than the correct figure, whilst those observed at p_H 6·3 may be slightly lower than the correct figure, the possible experimental error due to the method employed being comparatively large, whilst the actual difference in pressure over the range of p_H studied is very small.

Influence of the Concentration of the Gelatin on the Osmotic Pressure.

In the experiments just recorded a solution containing about 5 g. gelatin per 100 cc. was used throughout the investigation.

In order to discover whether the osmotic pressure is proportional to the concentration of gelatin in the solution a number of experiments have been made using gelatin solutions of different concentrations, viz. 1 %, 1·25 %, 2·5 % and 3·5 %. The gelatin was dissolved in a molar solution of sodium salicylate (as in the previous experiments).

It is found that the osmotic pressure of gelatin in these solutions is not proportional to the concentration of the colloid but increases very considerably with increased concentration of gelatin in the solution.

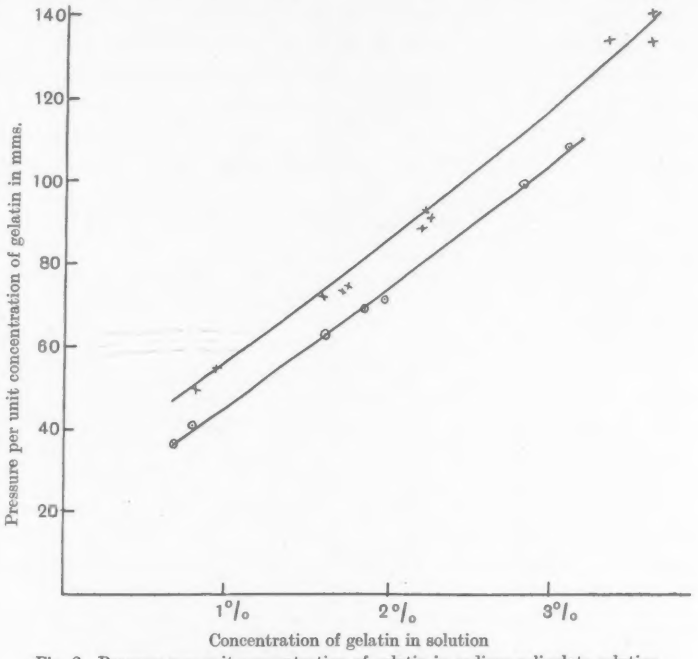

Fig. 2. Pressure per unit concentration of gelatin in sodium salicylate solution
⊙ 2*M* sodium salicylate × *M* sodium salicylate

The osmotic pressure of a 1 % solution of gelatin corresponds with an aggregate weight of 45,500 (if the usual formula be applied), whilst that of a 3 % solution corresponds with an aggregate weight of 16,500, the other concentrations employed giving intermediate figures as shown in the accompanying tables and curves (Fig. 2) (the curve showing the relation between the "pressure per unit concentration" and the concentration of the solution

indicates that the *increase* in pressure is proportional to the concentration of the solution).

The pressure within the osmometer (if started below the maximum obtainable for the solution under investigation) rises to a maximum, then falls gradually—as observed in the previous experiments. The results here given have been obtained by estimating the nitrogen in the solution at the conclusion of the experiment and assuming that the maximum pressure attained within the osmometer represents the osmotic pressure.

Some of the osmometers were filled to a greater height than the maximum obtainable when the initial pressure is low and in these cases the pressure fell rapidly until it agreed with the maximum pressure obtained by the first method of filling, then continued to decrease gradually as in the previous examples. As the point corresponding with the maximum obtained in the first type of osmometer cannot be readily estimated, no calculations of osmotic pressure (and corresponding aggregate weight) have been made in these cases.

Similar results were obtained when the gelatin was dissolved in $2M$ and $M/2$ salicylate solutions. In the latter case, owing to the tendency to gel formation, the readings are not very reliable. The osmotic pressures are somewhat higher in M than in $2M$ solutions (see Fig. 2).

Osmotic Pressure of Gelatin in Sodium Salicylate solution.

Nitrogen in 10 cc. solution mg.	Concentration of gelatin solution % (N = 18 %)	Pressure in mm. solution	Pressure per unit concentration of gelatin mm.	Temperature ° C.	Aggregate weight calculated from pressure
M sodium salicylate					
15	0·83	41	49·4	14	45,500
15·25	0·85	41	48·2	11	45,750
17·2	0·95	52	54·7	13	41,140
28·6	1·59	116	73	14	30,770
30·7	1·71	126	73·9	13	30,310
31·4	1·74	130	74·7	15	30,420
39·4	2·19	194	88·6	11·5	25,110
40·7	2·26	207	91·6	13	24,470
55·4	3·09	420	135	17	16,700
60	3·33	451	135	14	16,620
65	3·61	482	134	13	16,750
65	3·61	510	141	16	16,020
M/2 sodium salicylate					
33·1	1·84	116	63	14·5	35,690
34·1	1·91	139	72·8	14	30,890
35·2	1·95	136	69·7	15	32,420
13·07	0·726	32	44	14	51,100
14·43	0·8	40	50	14	45,130
2*M* sodium salicylate					
14·48	0·80	33	41·2	14	60,980
12·2	0·68	25	36·8	14	53,730
28·8	1·60	101	63·1	14	35,550
33·1	1·84	128	69·6	14·5	32,330
35·48	1·97	141	71·6	13	31,330
50·83	2·82	280	99·3	13	22,580
56	3·11	337	108·3	13	20,660

It is not possible with our present knowledge to explain the results obtained in this investigation, as so many factors such as solvation, salt-formation, and others which are perhaps not yet known, may have to be taken into account before any satisfactory generalisations can be made.

SUMMARY.

(1) The osmotic pressure of gelatin in sodium salicylate solutions has been determined.

(2) This depends on the concentration of the gelatin in the salicylate solution. The lower the concentration, the lower the osmotic pressure and the higher the apparent aggregate (or molecular) weight; thus in a 3 % solution in *M* sodium salicylate the apparent aggregate weight is about 16,000; this rises to about 40,000 in 0·9 % solutions.

(3) The osmotic pressure is not apparently influenced by small amounts of inorganic material in the gelatin.

REFERENCES.

Biltz, Bugge and Mehler (1916). *Z. physikal. Chem.* **91**, 705.
Briggs and Hieben (1920). *J. Physical Chem.* **24**, 74.
Loeb (1922). Proteins and the Theory of Colloidal Behaviour.
Knaggs, Manning and Schryver (1923). *Biochem. J.* **17**, 473.

CXLIV. SOME OBSERVATIONS ON THE EXTRACTION AND ESTIMATION OF LIPOCHROMES FROM ANIMAL AND PLANT TISSUES.

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(*Received August 1st, 1924.*)

During some recent investigations of the lipochrome content of various animal and plant tissues, certain observations made it seem desirable to examine some of the generally adopted methods of extraction, separation and estimation rather carefully. The results obtained may prove of use to other investigators in the same field.

It was hoped at first to adapt the simple method of grinding up the tissue with anhydrous sodium sulphate and extracting with light petroleum to a quantitative basis. The procedure adopted was as follows. The tissue to be examined was quickly cut into small pieces, a little ether was added to stop enzyme action, about 0·5 g. solid potash to decompose chlorophyll where present and the whole rubbed up with sand and anhydrous sodium sulphate. The dry mass was then extracted with light petroleum (B.P. below 40°) in a Soxhlet extractor for 2–3 days until no more pigment was taken out. The coloured extract was then evaporated down to a small bulk in a current of nitrogen, and submitted to Kraus' phase test. It was transferred to a stoppered measuring cylinder, an equal volume of 90 % methyl alcohol added, the mixture well shaken and the resulting volumes of the two liquids noted. (The total volume does not alter.) The carotene is dissolved almost completely in the upper layer of light petroleum and the xanthophyll in the lower layer of 90 % methyl alcohol. By separating these two liquids and further extracting each with a fresh portion of the other solvent, a very little pigment can be dissolved out of each phase. The second extracts so obtained appear to be about equally concentrated but in any case so dilute as to be unmeasurable by the only means (the Hellige colorimeter) at our disposal. Hence in subsequent operations the second extraction of each phase was not carried out. The total carotene and xanthophyll were calculated from the noted volumes and concentrations.

The only means at present available of estimating carotene or xanthophyll quantitatively is by comparing the given solution colorimetrically with one of known strength; or, as solutions of carotene and xanthophyll are unstable,

Willstätter proposed comparison with a solution of potassium dichromate which will keep indefinitely. A convenient strength to use is one of 0·2 % in water, but there is no straight line relation between the concentration of lipochrome solution and depth of colour of the dichromate solution. The very few data given by Willstätter and Stoll [1913] have been plotted graphically by Palmer [1922] for use with a Kober, Duboscq or Wolff colorimeter. As this is not directly applicable to readings obtained by the Hellige colorimeter, a fresh curve was made for this apparatus. The dichromate solution (0·2 %) is contained in a movable wedge of glass, the solution to be estimated in a stationary vessel whose front and back faces slope at the same angle as those of the movable wedge. These two vessels are so arranged in the apparatus that the colours are viewed side by side and the dichromate wedge can be raised until it matches the solution to be estimated. By starting with a strong solution of freshly crystallised carotene of known strength (about 2·5 mg. in 100 cc. light petroleum is suitable), making various dilutions and matching the dichromate solution to each one, a curve may be obtained by means of which any depth of carotene solution may be interpreted as milligrams of carotene per 100 cc. of solvent. The curve obtained for carotene is given in Fig. 1, p. 1121. It was confirmed by another worker (V. B. R.) in this laboratory and is practically identical with that obtained from a different sample of crystalline carotene.

Three preparations of xanthophyll crystals were made from dried nettle leaves by Jörgensen and Stiles' [1917] modification of the method used by Willstätter and Mieg [1907]. They served for spectrum and chromatographic analysis, but on attempting to recrystallise for an estimation curve, a brown oily liquid formed each time and fresh crystals could not be obtained. Hence, where necessary, the writer used the curve for xanthophyll very kindly lent to her by Dr O. Rosenheim.

The method of extraction of lipochromes detailed above gave such widely different results in total and relative amounts of carotene and xanthophyll extracted from different samples of the same material that certain points in the method were investigated.

In the first place, by working with a known amount of carotene in light petroleum solution, practically no lipochrome was recovered, and a similar loss was experienced in using sand and no anhydrous sodium sulphate. Potash seemed to destroy the whole of the carotene.

Adsorption on sodium sulphate was estimated by using known amounts of lipochrome and of the salt and quickly extracting in the usual way. The loss is shown in Table I, and is apparently proportional to the weight of sulphate used.

The variability in the ratio, carotene : xanthophyll, was investigated by extracting 50 g. etiolated wheat shoots prepared by the method already described and estimating the carotene and xanthophyll in successive fractions of the extract. From Table II it will be seen that the carotene is apparently

extracted earlier in the operation than the xanthophyll, which would account for a difference in the ratio of these two substances if the extraction had not been completed.

Table I.

	Anhydrous Na_2SO_4 g.	Vol. of sol. cc.	mg. carotene in this vol. of sol.	mg. carotene recovered	Total loss	% loss
A	5	5	0·10625	0·0875	0·01875	17·6
B	10	5	0·10625	0·0712	0·03505	33·0
	Repeated with a weaker solution.					
C	25	25	0·06	0·0475	0·0125	20·8
D	50	25	0·06	0·035	0·025	41·7

Table II.

	Estimated at end of	mg. carotene obtained	mg. xanthophyll obtained	Ratio $C:X$
1st fraction of extract	24 hrs	0·0036	0·0112	0·32
2nd " "	28 "	0·0028	0·0133	0·21
3rd " "	72 "	0·0023	0·0132	0·17
4th " "	120 "	0·00167	0·017	0·098

Hence the method of grinding up with potash and anhydrous sodium sulphate and extraction in a Soxhlet was discarded for quantitative work.

Saponification with *alcoholic* potash and subsequent extraction does not give quantitative results, as much yellow resinous material is formed and extracted by the light petroleum. Saponification with *aqueous* potash however gives satisfactory results, though in this case it is necessary to add about 20 cc. alcohol to the product before extraction, otherwise no lipochrome at all is extracted. The whole process must also be carried out as nearly as possible in an atmosphere of nitrogen. To compare the influence of different strengths of potash solution, 10 cc. of a light petroleum solution of carotene (containing 0·115 mg. carotene) together with 5 g. dried egg albumin (to imitate somewhat the conditions of an ordinary saponification) were saponified with 200 cc. of potash solutions of 50, 25, 18·75, 12·5, 6·25, 2·5 % respectively. The extracts were made up to 10 cc. the original volume of lipochrome solution. The Hellige reading of the original solution was 55·6, those of the extracts 56·4, 55·7, 55·6, 55·5, 55·4, 55·5 respectively (each an average of nine readings). The first indicates a loss of 0·003 mg. in 0·115 mg. carotene and was obtained with unnecessarily strong potash. The rest may be regarded as quantitative results.

Tswett's Chromatographic Method of Analysis of Lipochromes.

Tswett [1906, 2] claims to have separated four different xanthophylls from the pigments of green leaves by his chromatographic analysis. He names them α, α', α'', β respectively and gives their spectra as:

	Band I	Band II	Band III
Xanthophyll α (nearest the base)	485–470	455–440	—
Xanthophyll α'	Slightly shifted towards the violet		
" α''	"	"	"
" β	475–462	445–430	—

From his wide investigations of the adsorptive powers of different substances (some 30–40) for these pigments and for other substances, and also from the fact that his four xanthophylls give different spectra, Tswett [1906, 1] assumes that they are definitely different substances. He appears, however, to have examined the lipochromes of green or yellowing leaves only, by this method. Hence it seemed desirable to examine other tissues by this method as they might give a greater or smaller number of xanthophylls or different lipochromes altogether.

The perianths of the polyanthus narcissus were first examined and were found to be such a convenient and rich source of the lipochromes that this material was used for testing several points in the principle of the chromatographic method. Ten single flowers gave a solution strong enough for the purposes of a chromatograph. The tubes of the perianths were discarded as they were somewhat green. The tissue was ground up with anhydrous sodium sulphate and quickly extracted (but never completely so) with cold light petroleum. The chromatograph was made at once and washed thoroughly with the pure solvent. The column was removed, the layers separated and extracted with ether. The solutions gave the following spectra:

	Band I	Band II	Band III	Substance presumably
Filtrate	489–469	454–439	425 to end	Carotene
Layer (*a*) near base	482–467	451–436	425 „	Xanthophyll α
„ (*b*)	480–465	449–434	422 „	„ α'
„ (*c*)	478–465	449–434	221 „	„ α''
„ (*d*) near top	476–464	447–431	420 „	„ β
Size of slit of spectroscope (mm.)	0·08 0·08	0·08 0·095	0·12	

In each case the spectrum was read several times, an average made and the result expressed to the nearest $\mu\mu$.

This experiment has been repeated several times, each time with similar results. Incidentally, it was found that in using a strong solution of the lipochromes, xanthophyll α was easily retained in the chalk but that the other three xanthophylls were not very widely separated. On the other hand, by using a weak solution, the xanthophylls α', α'' and β were more widely separated but xanthophyll α was washed out into the flask together with, or just behind, the carotene. By passing this mixture of xanthophyll α and carotene through a fresh chalk column in more concentrated solution, they were conveniently separated. Also, after thorough washing of a chromatograph with the pure solvent (light petroleum was used throughout in these experiments) any one well-defined layer could be extracted with ether, evaporated in a current of nitrogen, taken up in light petroleum and another chromatograph attempted with it. But in each case, only one layer was obtained and the filtrate was colourless. Furthermore, on combining the extracted, evaporated and redissolved pigment from any one layer with the filtrate from the original chromatograph, only one layer was formed in a fresh chromatograph and the fresh filtrate was coloured.

Thus it would appear that the four xanthophylls really are different substances, separated as Tswett suggested by their different adsorption affinities for the chalk.

The lipochromes of a very deeply-pigmented daffodil, "King Alfred," have also been examined in this way, and four distinct layers obtained. Their spectra were:

	Band I	Band II	Band III	
Xanthophyll α	482–467	449–436	418? to end	
„ α'	478–465	448–434	417 „	Possibly the same xanthophyll
„ α''	478–464	446–433	416 „	
„ β''	476–460	444–432	418? „	
Size of slit of spectroscope (mm.)	0·08 0·08	0·08 0·095	0·12	

This material also was used to demonstrate the fact that the lipochrome of a single layer from one chromatograph gave only a single layer on passing through a fresh chalk column and that the lipochrome of any one layer combined with the filtrate gave only one layer and a coloured filtrate on attempting a fresh chromatograph.

Three occurrences of a very curious phenomenon have been observed during the making of some forty or fifty chromatographs. Practically always the four xanthophylls may be depended upon to take up their positions as indicated, but three times xanthophyll β, identified by its spectrum, has taken up a position below xanthophyll α'', and once this was seen to happen during the washing when it had already been stationary for some little time at the top of the column. Whether a different sample of light petroleum was used at this point is not recorded.

Crystalline xanthophyll, prepared by the method of Jörgensen and Stiles and containing some sterol, dissolved readily in light petroleum. Its solution gave the spectrum 482–465, 450–435, 425 to end. On attempting a chromatograph, only one layer was obtained which widened slightly on prolonged washing. This was dissolved in ether and the solution gave a spectrum 482–466, 450–434·5, 425–end. On repeating with a weaker solution of crystalline xanthophyll, the following spectra were obtained:

	Band I	Band II	Band III
Before chromatographic analysis	482–467	451–435·5	424·5 to end
After „ „	482–467	452–436	425 „

A Separation of Lycopin and Carotene.

While investigating various sources of lipochromes for Tswett's four xanthophylls, the corollas of the ray florets of the African marigold (*Tagetes erecta*) were examined. A light petroleum extract of this tissue was passed through a chalk column in an attempt to get a chromatograph. The coloured filtrate first obtained was pinkish-yellow and a second and third fraction of the filtrate were a similar colour but the fourth was distinctly more greenish-yellow than the previous ones. This suggested the possibility that some lipochrome might have passed through the column more easily than the carotene. Spectrum analysis of the fractions gave the following results:

	Band I	Band II	Band III	Band IV	Lipochrome
1st fraction of filtrate	500–483	467–453	428–419	413 to end	Some lipochrome other than carotene
2nd " "	490–479	452–440	428 to end	—	Carotene
3rd " "	491–481	452–440	428 "	—	"
4th " "	*	453–441	428 "	—	"

* Absent even on concentrating considerably.

Various observers report the following readings of spectra of lipochromes:

Lipochrome	Band I	Band II	Band III	Solvent	Observer
Lycopin	510–499	480–468	440 to end	Alcohol	Willstätter and Escher (1910)
Carotene	492–478	459–446	—	"	Willstätter and Stoll (1913)
"	490–475	455–445	430–418	"	Kohl (1902)
"	490–475	455–445	430–418	Ether	"
"	492–475	460–445	—	Light pet.	Tswett (1911)
"	488–470	456–438	—	Alcohol	Willstätter and Mieg (1907)

The lipochrome other than carotene observed in the filtrate from the extract of pigments from the African marigolds does not appear to be lycopin. It gave the typical colour reactions for lipochromes with concentrated sulphuric and nitric acids but no further means were taken to determine its nature. The interest of the observation lay in the suggestion of a possible means of separation of two lipochromes such as lycopin and carotene.

This experiment was repeated with a light petroleum extract of tomatoes dried with anhydrous sodium sulphate. The chief lipochrome of this fruit is lycopin. On passing through a chalk column, the first fraction of the filtrate was a pure yellow, the second fraction taken was slightly pink, the third distinctly pink, and also the fourth. Left in the chalk were two sharp yellow bands near the top of the column, and a wide very diffuse pink part which would probably have passed out completely on further washing. The spectra of these parts were:

	Band I	Band II	Band III	Lipochrome
1st fraction of filtrate	487–471	453–440	424 to end	Carotene
2nd " "	487–471	453–440	427 "	"
3rd " "	507–490	476–457	443–433	Lycopin
4th " "	507–491	477–458	444–432	"
Lowest part of column	508–492	478–459	444–432	"
Middle " "	508–493	479–461	Unreadable	"
Lower yellow zone	482–466	453–442	430 to end	Xanthophyll α
Upper "	476–462	448 to end	Unreadable	" β

To investigate this further as a possible means of separation of lycopin and carotene, solutions in light petroleum of the substances, each twice recrystallised, were made and mixed in such proportions that the spectrum analysis gave:

	Band I	Band II	Band III
Pure lycopin solution	509–492	479–461	447–435
Pure carotene solution	489–471	456–441	428 to end
Mixture of the above—dilute	489–462	454–436	427 "
Mixture of the above on concentrating	508–492	Rest indistinguishable	

This solution was then passed through a chalk column with suction, as if for a Tswett's chromatograph. The filtrate became coloured before the washing with the pure solvent was begun. This point may be of importance in view of

a later experiment. The filtrate was removed at very short intervals, about 2 cc. being collected each time. The fractions were then examined spectroscopically, being diluted where necessary so that they were of comparable strength.

Fraction of filtrate	Band I	Band II	Band III	Lipochrome
I*	489–473	455–440	425 to end	Carotene
II	489–470	454–438	426 ,,	,,
III	489–470	455–438	426 ,,	,,
IV	489–467	455–440	427 ,,	,,
V	489–468	454–438	425 ,,	,,
VI	489–468	455–439	427 ,,	,,
VII	489–470	455–440	429 ,,	,,
VIII	Very faint trace	487–462	448–437 426–	Carotene + lycopin
IX	509–491	477·5–460	446–436	Lycopin
X	508–493	480–462	447–435	,,
XI	509–491	478–461	447–433	,,
XII	509–492	478–460	447–434	,,

* Very weak solution.

Fractions I–VII were then combined and gave the spectrum:

489–468, 455–438, 428 to end,

but no trace of the first lycopin band even on concentrating so much that the carotene bands could not be read. This solution was filtered through a fresh chalk column and gave the following fractions:

Fraction of filtrate	Band I	Band II	Band III	Lipochrome
I	488–470	455–440	429 to end	Carotene
II	489–468	455–438	427 ,,	,,
III	489–468	454–439	428 ,,	,,
IV	489–468	454–441	429 ,,	,,
V	510–491	478–462	449–435	Lycopin

Fractions IX–XII of the first filtration were then combined and gave 509–490, 477–463, 449–436 which on concentrating gave 510–492, 479–460, 450–436, so that no evidence of the presence of carotene was found by this means. It was then filtered through a fresh chalk column, and five fractions of the filtrate were taken.

Fraction of filtrate	Band I	Band II	Band III	Lipochrome
I	489–471	456–439	426 to end	Carotene
II	488–468	453–440	427 ,,	,,
III	509–491	477–461	447–434	Lycopin
IV	509–490	477–459	447–433	,,
V	489–470	455–440	430 to end	Carotene

In this experiment it should be noted that washing with the pure solvent had begun long before the coloured solution had begun to emerge from the tube. The possible significance of this point will be taken into consideration in further work which is contemplated, and may serve as an indication for an explanation of the above observations.

Readings of scale of Hellige colorimeter

With the exception of the last isolated observation, it had always been rather difficult to remove the last traces of lycopin from the chalk column, though prolonged washing had achieved it. Its removal by ether was immediate and apparently complete.

Fractional filtration, of which an example has been described above, suggests a possible method of separation of closely allied substances, if the adsorbent suitable to the particular case can be found.

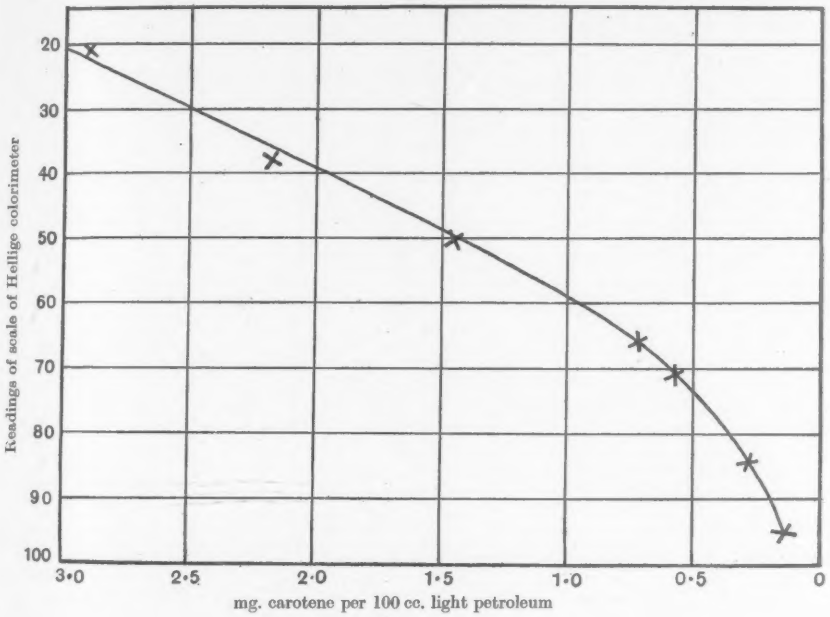

Fig. 1

Summary.

1. Several points in generally adopted methods of extraction of lipochromes have been examined and the results show these methods to be unsuitable for quantitative estimations of the pigments.

2. The only method of extraction giving quantitative results was saponification with aqueous potash of strength not greater than 20 %. Each step of the process should be carried out in an atmosphere of nitrogen as far as possible.

3. A curve for use with a Hellige colorimeter is given for the estimation of carotene.

4. Tswett's chromatographic method of analysis of lipochromes has been examined on many points. It appears to support his theory of the existence of four xanthophylls.

5. Fractional filtration through a chalk column has been found to be suitable for separation of carotene and lycopin from a mixture of the two substances. It may prove useful for the separation of other closely related substances.

The writer would express her gratitude to Professor J. C. Drummond for his helpful advice and criticism on this work and to the Royal Society and the Medical Research Council for grants which defrayed the cost of the investigation.

REFERENCES.

Jörgensen and Stiles (1917). Carbon Assimilation, *New Phytologist Reprint*, No. 10.

Kohl (1902). Untersuchungen über das Carotin und seine physiologische Bedeutung in den Pflanzen (Leipzig), Chap. III.

Palmer (1922). Carotinoids and Related Pigments (Chem. Catalog Co., New York), 260.

Tswett (1906, 1). *Ber. botan. Ges.* **24**, 316.

—— (1906, 2). *Ber. botan. Ges.* **24**, 384.

—— (1911). *Ber. botan. Ges.* **29**, 630.

Willstätter and Escher (1910). *Z. physiol. Chem.* **64**, 47.

Willstätter and Mieg (1907). *Liebig's Annalen*, **355**, 1.

Willstätter and Stoll (1913). Untersuchungen über Chlorophyll (Springer, Berlin).

CXLV. THE LIPOCHROMES OF ETIOLATED WHEAT SEEDLINGS.

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(*Received August 1st, 1924.*)

The association of vitamin A with some lipochrome (generally carotene) in all recorded cases of its occurrence in plant tissues [Steenbock and Gross, 1919; Steenbock, Sell and Boutwell, 1921; Steenbock and Sell, 1922; Coward, 1923] and the rapidity with which wheat seedlings grown in the dark form vitamin A on exposure to light, made it seem desirable to make a careful examination of the pigments occurring in these etiolated shoots.

The formation of a yellow pigment in small grains in the cells of plant structures grown in the dark has long been recognised. The pigment was known to be soluble in alcohol and was called "etiolin" by Pringsheim [1874] because it occurred in etiolated structures. Willstätter and Stoll [1913] identify "etiolin" with carotene of carrots, etc., on the strength of the similar solubilities and crystal form of the two substances. Other workers report various findings and employ various terms to designate the pigments. This has given rise to considerable confusion in the literature, a summary of which is given by Palmer [1922]. The term "etiolin" is now obsolete although shoots grown in the absence of light are still spoken of as "etiolated." It is proposed to give here the results of an investigation of the lipochromes of etiolated wheat seedlings in the light of the later work on lipochromes of Willstätter and his co-workers and of Tswett [1906, 1911].

The seeds used throughout the experiments were Carter's "Red Stand-up" variety. No detectable traces of lipochromes were found in them, even on saponifying and extracting 100 g. They were germinated in ordinary garden mould mixed with about a quarter of its weight of sand, in boxes in a large airy dark room at a fairly uniform temperature of 6–10°. They were allowed to grow for 17 days and the shoots used in the investigation were cut immediately below the first outstanding leaf.

The pigments of the etiolated shoots were roughly identified as lipochromes by grinding up the tissue with anhydrous sodium sulphate, extracting with ether and applying the usual tests. Carotene and xanthophyll were both identified, the latter being present largely in excess of the former.

For reasons explained in the preceding paper, the quantitative estimation of the lipochromes was made, after saponification of the tissue with about

twice its weight of 20 % aqueous potash for 3 days, by addition of alcohol which seems to liberate the pigments, and extraction with light petroleum (B.P. below 40°). The extract was washed six times with water but not dried with anhydrous sodium sulphate. Apparently light petroleum takes up only a very small quantity of water, and this would not interfere materially with the estimation. The extract was evaporated down in nitrogen, an aliquot part taken, the carotene and xanthophyll separated by the addition of an equal volume of 90 % alcohol as in the Kraus phase test, the resultant volume of each phase noted, and the concentration of each estimated by the Hellige colorimeter. In this way 1·97 mg. carotene and 10·3 mg. xanthophyll were obtained from 400 g. fresh shoots.

Chromatograph of lipochromes of etiolated wheat seedlings.

It was attempted to make a chromatograph of the lipochrome solution obtained by the saponification of 400 g. fresh shoots as detailed above. This was found to be impossible, a certain amount of pigment being retained in the chalk, but most of it coming through in the filtrate.

Other attempts were equally unsuccessful and hence it must be concluded that the very long saponification, although carried out in nitrogen, had influenced the lipochromes somehow. A quick saponification of a smaller quantity (about 20 g. for 6 hours) gave a quite good chromatograph whose different layers gave the following spectra in ethereal solution.

	Band I	Band II	Band III	Substance presumably
Filtrate	489·5–472	455–442	428 to end	Carotene
Layer 1 (at base)	485 –472	453–440	426 ,,	Xanthophyll α
,, 2	481 –468	452–438	424 ,,	,, α'
,, 3	478 –467	450–438	424 ,,	,, α''
,, 4 (at top)	476 –465	448–436	422 ,,	,, β
Size of slit of spectroscope	0·08 0·08	0·08 0·095	0·12 mm.	

Apparently the four xanthophylls identified by Tswett [1906, 1911] in green leaves are also present in etiolated ones.

A fairly complete extraction can also be made by grinding up the fresh material with sand and alcohol, but as alcoholic solutions pass straight through the chalk column, the solution has to be filtered, evaporated to dryness in nitrogen under reduced pressure and then taken up in light petroleum. With the greatest care this cannot be really quantitative as there must be partial loss of one or more constituents by incomplete extraction and during the evaporation to dryness. A solution of the lipochromes of etiolated wheat seedlings made in this way gave four distinct layers in a chromatograph and a coloured filtrate.

The solutions of the layers of lipochromes from many chromatographs have been examined spectroscopically. The results given above are typical but slight variations have been noticed. When, however, it is realised that it is almost impossible to get solutions of the same strength for purposes of

comparison and that the edges of the bands are very ill-defined, the discrepancies may be pardoned. Tswett [1911] suggests that the slightly different spectra of carotene recorded by different observers may indicate the existence of "different homologous carotenes" but the differences appear to be only such as might result from the difficulties mentioned above.

The solutions of the four xanthophylls from etiolated wheat seedlings described above and identified spectroscopically were combined and concentrated. The spectrum of the solution was then 484–465, 452–434, 426 to end, which appears to be a simple combination of the four individual xanthophylls.

It was originally intended to make an estimation of the relative proportions of the four xanthophylls in etiolated wheat seedlings. Tswett mentions the desirability of investigating his chromatographic analysis quantitatively but does not appear to have done so.

With this purpose in view, the four layers of a chromatograph of lipochromes from etiolated wheat seedlings were separated. Each part was dissolved out with ether, filtered and the residue washed as completely as possible; the volume of the filtrate was noted and the concentration estimated by the Hellige colorimeter. The whole solution was evaporated just to dryness in a test-tube in a current of nitrogen, dissolved in light petroleum and the solution made up to its original volume. Its concentration was again determined to estimate loss by heating and drying, it was passed through a fresh chalk column, dissolved out again with ether as completely as possible and the solution again made up to its original volume. The concentration of this was estimated to see whether the extraction from the chalk had been complete. Even with the greatest care, there was loss of lipochrome in each process as may be seen from the following table.

		First extract from chalk		Light pet. solution		After second filtration		
		Vol. cc.	H.R.	Xanth. mg.	H.R.	Xanth. mg.	H.R.	Xanth. mg.
Xanth.	α	5·3	76·5	1·99	84·5	1·06	96·5*	0·1325
,,	α'	6	82·3	1·35	90·0	0·6	92·6	0·36
,,	α''	3	88·5	0·375	91·5	0·225	95	0·12
,,	β	4	82	1·0	86·3	0·6	91	0·3

Bulk of chalk in second filtration			
Greatest,	about	1 inch	depth
Least,	,,	0·15 ,,	,,
	,,	0·2 ,,	,,
	,,	0·2 ,,	,,

* Too dilute a solution for an accurate comparison.

Apparently the greater the bulk of chalk over which any one xanthophyll solution spreads itself, the greater the loss of lipochrome in the process. No strict comparison is possible in these figures as neither the total xanthophyll nor the concentration of the different solutions used was equal. In any case, as these factors cannot be controlled in the making of a chromatograph, it seems to be impossible to make a quantitative comparison of these lipochromes by this method.

SUMMARY.

1. The pigments of etiolated wheat seedlings have been identified with the carotene and four xanthophylls isolated by Tswett from green leaves.

2. It is not possible to make a quantitative estimation of the four xanthophylls separated from each other by Tswett's chromatographic method.

The writer would express her sincere thanks to Professor J. C. Drummond for his kind advice and criticism on the work reported in this paper, and to the Royal Society and the Medical Research Council for grants from which the cost of the work was defrayed.

REFERENCES.

Coward (1923). *Biochem. J.* **17**, 145.
Palmer (1922). Carotinoids and Related Pigments (Chem. Catalog Co., New York), 48.
Pringsheim (1874). *Monatsh. Akad. Wiss. Berlin*, 628.
Steenbock and Gross (1919). *J. Biol. Chem.* **41**, 149.
Steenbock and Sell (1922). *J. Biol. Chem.* **51**, 65.
Steenbock, Sell and Boutwell (1921). *J. Biol. Chem.* **47**, 303.
Tswett (1906, 1). *Ber. botan. Ges.* **24**, 316.
—— (1906, 2). *Ber. botan. Ges.*, **24**, 384.
—— (1911). *Ber. botan. Ges.* **29**, 630.
Willstätter and Stoll (1913). Untersuchungen über Chlorophyll (Springer, Berlin).

CXLVI. THE COLORIMETRIC ESTIMATION OF LYCOPIN.

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(*Received August 1st, 1924.*)

PALMER [1922] states that "No method has yet been devised for estimating lycopin quantitatively. A suitable standard must first be found, and a method discovered for separating lycopin from carotene." It has recently been shown by Coward [1924] that it is possible to separate lycopin from carotene by filtration through a column of dry chalk, and it only remained to find a suitable colour standard.

A number of easily purified inorganic salts were examined but none was found whose colour changes, on successive dilution, matched those of lycopin. It was therefore decided to adopt the dilution colorimeter of Stanford [1923] and to make up an artificial standard whose colour and intensity should correspond to that of a dilute solution of lycopin of known concentration.

A crystalline sample of lycopin was prepared from fresh tomatoes by the method described by Willstätter and Escher [1910]. A portion of this was dissolved in light petroleum (B.P. below 40°) and its purity tested by the method of chromatographic fractionation as described by Coward [1924]. No trace of any other colouring matter could be detected spectroscopically in any of the fractions.

The strength of lycopin solution chosen was 0·5 mg. per litre, which was considered to be as dilute as any which would be obtained in analytical experiments. A few milligrams of the lycopin were therefore weighed out by means of a micro-balance, with an accuracy of 1 part in 200, and dissolved in the amount of light petroleum necessary to make a solution of this strength. A layer of this solution 30 mm. thick is exactly matched by a standard solution prepared in the following way. Two stock solutions are made up (1) potassium dichromate 0·02 %, (2) cobalt sulphate (nickel free) 0·5 %. Of these, quantities equivalent to (1) 6·9 mg. and (2) 135·0 mg. respectively are taken, mixed, and the volume made up to 100 cc. This standard is stable for at least 24 hours, and the two solutions can be kept separately for a considerably longer period.

The apparatus used is essentially that of Stanford, the source of light being a Sheringham Daylight Lamp, and the comparison eyepiece that of a Duboscq colorimeter. Since the solvent used is light petroleum it is advisable to use

plain open cells as described by Stanford. A rectangular glass cell 30 × 30 × 100 mm. high was therefore obtained, and graduated on one side to measure up to 80 cc. A thick (8 mm.) plate glass lid was made for this, centrally drilled and a stopper ground in. The lid is held in place by rubber bands, the joint being smeared with a little glycerol which, since it is insoluble in light petroleum, prevents leakage. For the standard solution a plain cell 30 × 30 × 30 mm. is used[1].

In order to carry out an estimation, the lycopin solution of unknown strength is diluted in a measuring cylinder until it is only slightly darker than the standard, and the degree of dilution noted. 25 cc. of this are then measured into the glass cell and light petroleum added until the two solutions match. The volume of the solution is then read off. Each cubic centimetre of liquid in the cell contains 0·0005 mg. of lycopin, and from this reading and a knowledge of the previous dilution the total lycopin present is calculated.

A series of tests with lycopin solutions of various strengths has shown that the concentration of 0·5 mg. per litre, arbitrarily chosen, is actually more easily compared than those which are either stronger or weaker than this.

It has been found possible for a practised observer to carry out a series of estimations with an error of considerably under 1 %.

I am indebted to Miss Katharine H. Coward for suggesting this piece of work and for help in determining the colour standard.

[1] The glass cells used in this estimation were obtained from Messrs J. J. Griffin and Sons, Ltd., Kingsway, London.

REFERENCES.

Coward (1924). *Biochem. J.* **18**, 1114.
Palmer (1922). The Carotinoids and Related Pigments (Chemical Catalog Company, New York), 261.
Stanford (1923). *Biochem. J.* **17**, 839.
Willstätter and Escher (1910). *Z. physiol. Chem.* **64**, 47.

CXLVII. INVESTIGATION OF BARLEY, MALT AND BEER FOR VITAMINS B AND C.

By ARTHUR HARDEN and SYLVESTER SOLOMON ZILVA.

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(*Received July 21st, 1924.*)

Some years ago [1918] we had the opportunity of investigating beer, malt and barley for their antineuritic and antiscorbutic potencies. The development of the study of vitamins during the intervening period has made it necessary to repeat and amplify our inquiry on this subject and this supplementary investigation forms the subject of the present communication.

BARLEY AND MALT.

(*a*) Vitamin B.

In our original investigation we found that when malt was given to pigeons as the sole source of the antineuritic factor the birds grew but eventually developed polyneuritis. This experiment has now been repeated on pigeons as well as on rats.

Experiments on Pigeons.

In the bird experiment not only was the malt tested out but the original barley and the grain in the intermediate stages of the malting process were also investigated.

For this purpose Mr E. H. Ludlow of the Mortlake Brewery (Messrs Watney, Coombe, Reid & Co.) was kind enough to arrange throughout the period of the experiment (12. xii. 21–14. ii. 22) a continued series of maltings of a Chilian barley, so that fresh samples of the intermediate stages of the malting process could be obtained at intervals of 2 days. The intermediate stages selected were (*a*) after steeping, (*b*) after 5 days on the floor, (*c*) just before kilning, and in addition the original barley, the finished malt and the culms were examined.

Six pigeons were fed on the grain of each of the above stages, which was given to them *ad lib.* The birds on the steeped grain, 5 days germinated grain, and the germinated grain before kilning throve, gained in weight, and failed to develop polyneuritis after 2 months. The pigeons on the barley and on the malt did not gain in weight but were also protected from the disease. The group of birds on the culms alone died of polyneuritis within a month, manifesting a loss of weight.

As these results differed from that previously obtained with regard to the antineuritic properties of malt, a number of other samples of malt, dried off at various temperatures, were also tested on pigeons.

We are also indebted to Messrs Watney, Coombe, Reid & Co. for these samples.

They were marked:

Malt	Kilning temperature
(1) English Pale	195° F.
(2) „ K.K.K.	216°–220° F.
(3) Californian	195° F.
(4) English Amber	216°–220° F.
(5) „ X	200° F.

Six pigeons were fed *ad lib.* on each of these samples; nearly all gained in weight, all throve well, and did not develop symptoms of polyneuritis in 3 months, after which the experiment was terminated.

Experiments on Rats.

Barley and malt were also tested on rats. Sets of six animals were placed on diets free from the antineuritic factor (vitamin B) containing 10, 20, 30 and 50 % barley or malt respectively (3. iv. 23–1. vi. 23). The materials used were Californian barley and the malt prepared from it. These were also kindly supplied by Messrs Watney, Coombe, Reid & Co. The vitamin-free diet was compounded as follows:

Basal mixture	55 %
Purified butter fat	10
Decitrated lemon juice	27
Hardened cotton seed oil	8

The basal mixture consisted of 20 % purified casein (extracted with hot 90 % alcohol), 75 % of starch and 5 % salt mixture. Six control rats on this diet deteriorated and succumbed to the deficiency within a month.

The rats in every group receiving the malt gained in weight and survived for 2 months, after which time the experiment was terminated. The animals in the 10 % group gained on the average 25 g. during the period, those in the 20 % 30 g., in the 30 % 50 g. and in the 50 % 80 g.

Four groups of rats (six animals in each group) receiving the basal diet together with admixtures of 10, 20, 30, 50 % of the original barley from which the malt was made behaved in precisely the same manner as those animals which received the malt.

The pigeon and rat experiments both show that malt as a rule is decidedly active. It appears, however, that the antineuritic activity of malt may depend on the sample since in our original investigation no such activity could be established.

(*b*) Antiscorbutic factor (vitamin C).

In our original communication we found that malt does not possess any antiscorbutic activity, and since barley on germination acquires such activity [Fürst, 1912] we thought it advisable to find at what stage of the malting process it first appears and when it disappears. We, therefore, examined the grain for the antiscorbutic factor in the same stages of the process as we used for the antineuritic factor. Six guinea-pigs were used in each group, and were placed on an *ad lib.* ration of the grain and 40 cc. of autoclaved milk per day. As was to be expected, the barley and malt were found totally inactive. The culms had a toxic action on the animals and, therefore, the results from this group were inconclusive. The animals on the grain after steeping, after 5 days on the floor, and just before kilning, throve well on the diet and were totally protected from scurvy for 2 months, after which time the experiment was terminated. The guinea-pigs on the active grains consumed more food than those on the barley and the malt (about 15 g. per day) but when one takes into consideration the difference in the moisture content this disparity in consumption of the food is not very great and could in no way influence the results obtained. That the barley should become active so early in the process as after the steeping is very striking.

BEER.

(*a*) Antiscorbutic factor (vitamin C).

We previously found that fined beer did not contain the antiscorbutic and antineuritic factors. In testing it for the antiscorbutic factor the beer was fed to guinea-pigs both liquid and as dry residue. It was found that it did not prevent or even delay the onset of scurvy in these animals. Fresh beer was also tested on a monkey subsisting on a scorbutic diet and in this case as well the animal succumbed to scurvy after 4 months. A great number of monkeys have since been employed in this laboratory for testing the antiscorbutic activity of various substances and in no case was it found that the period required to induce scurvy in these animals was longer than about 10–12 weeks. It was, therefore, thought desirable to repeat the beer experiment on a monkey in order to ascertain whether the somewhat unusual delay observed in our first experiment in the onset of the disease was due to any antiscorbutic activity of the beer. Beer (India Pale Ale) was therefore again administered to two monkeys (*Macacus rhesus*) subsisting on a scorbutic diet which consisted of rice, casein, marmite and additional salts with the occasional addition of cod liver oil. 200 cc. of the beer was offered daily. The quantity consumed by the animals varied but the average consumption was about 150 cc. per day. One animal showed well-defined symptoms of scurvy after 37 days, the other after 57 days. This definitely confirms the observation that no appreciable antiscorbutic potency is present in the beer we have examined.

(*b*) Vitamin B.

Our previous experiments with pigeons had shown that the residue obtained by evaporating beer under diminished pressure had no antineuritic potency. A further test has now been made on the effect of a similar residue on the growth of rats. About 20 gallons of India Pale Ale, supplied by the kindness of Messrs Watney, Coombe, Reid & Co., was evaporated at 65–70° in a small vacuum pan. Particular care was taken that the beer should be bright and free from yeast, as the latter of course is known to be rich in vitamin B.

Five groups of rats of six animals in each set were employed. The constitution of the vitamin-free diet was the same as that which we employed in the tests carried out on the malt. 5, 10, 15, 20 and 25 % of dried beer was incorporated in the diet of the respective groups. The first three groups of animals all succumbed within 4 weeks. The two last groups lived a little longer, namely, about 5 weeks. This slight delay could not be attributed to the presence of any traces of the vitamin since some of the control animals subsisting on the basal diet alone also lived as long.

This confirms our previous result that the beer examined by us did not contain vitamin B.

Summary.

1. The vitamin B of barley is not affected by the malting process and is frequently present in the finished malt, but is absent from the culms.

2. The antiscorbutic vitamin appears in the steeped grain and is present in the green malt but absent from kilned malt.

3. The beer examined by us was free from both vitamins B and C.

Our thanks are due to the Directors of Messrs Watney, Coombe, Reid & Co. for their kindness in supplying the materials employed and to Messrs J. L. Baker and E. H. Ludlow for their personal assistance. Our thanks are also due to the Medical Research Council for a whole time grant to one of us.

REFERENCES.

Fürst (1912). *Z. Hyg. Infekt.* **72**, 121.
Harden and Zilva (1918). *J. Inst. Brewing*, **24**, 197

CXLVIII. CHANGES IN THE PHOSPHORUS PARTITION IN HUMAN BLOOD DURING AMMONIUM CHLORIDE ACIDOSIS.

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(*Received August 1st, 1924.*)

Phosphorus occurs in at least four forms in human blood: inorganic phosphate, two forms of organic acid-soluble phosphoric esters, one of which is hydrolysed by an enzyme occurring in bone [Kay and Robison, 1924] and in other tissues, and "lecithin." Bloor [1918] showed in 1918 that the acid-soluble portion of protein-free blood filtrates and the portion soluble in ether-alcohol contained between them all the phosphorus, i.e. that no phospho-protein is present. In determining, therefore, the distribution of phosphorus at any given time between these four groups, although there may be more than one substance present in any one group, no phosphorus-containing compound of importance is neglected.

The acid-soluble portion, in which there are at least two, and possibly four, different phosphorus-containing substances, contains among these only one compound—adenine nucleotide—which has up to the present been identified with any degree of certainty [Jackson, 1924]. It is nevertheless feasible, even with the meagre knowledge already acquired, to give some answer to the question as to the possible functions of these unidentified phosphorus compounds, and there seems to be no doubt, for example, that bone formation is as intimately concerned with one of the organic phosphoric esters present in blood [Kay and Robison, 1924; Robison and Soames, 1924] as is fat metabolism with the ether-soluble phosphorus compounds [Bloor, 1916].

From the experiments of Haldane, Wigglesworth and Woodrow [1924] on J. B. S. H. it is known that ammonium chloride acidosis leads to a marked disturbance of the phosphorus partition in blood, and it was with a view to obtaining further evidence as to the possible functions of these different phosphorus compounds that advantage was taken of a recent experiment[1] in this laboratory in which the same subject ingested large quantities of ammonium chloride on each of three successive days, to examine this disturbance in more detail.

For the analyses, blood was taken from an arm vein each morning about half an hour after rising, while the subject was still fasting. Beginning in the

[1] Other results of this experiment will shortly be published.

morning of the day during which the first 25 g. of ammonium chloride were taken, these analyses were continued for 6 days after the last ammonium chloride day, until the inorganic phosphorus had returned to its normal concentration. When the daily blood analyses were discontinued, the amounts of the other phosphorus compounds in the blood had almost, but not quite, reached their normal level, and when a final blood sample was taken 4 days later the correspondence between the first (normal) day sample and this last sample was complete, i.e. the phosphorus distribution had returned to the normal.

The inorganic phosphorus was determined by the Briggs method; the total acid-soluble phosphorus by ignition with a sulphuric-nitric acid mixture followed by the same colorimetric procedure; the "lecithin" phosphorus by what was practically the method of Randles and Knudson [1922] and the "hydrolysable" phosphorus by the method of Kay and Robison using enzyme freshly prepared from rat bone. The blood filtrates for the hydrolysable phosphorus determination were neutralised, a little chloroform added, and then placed in the cold chest at 0°. There they remained until the end of the experiment, when all were treated together under strictly comparable conditions with the bone enzyme. It is known from previous work that keeping the blood filtrate at 0° for a much longer period has no effect on the hydrolysable phosphoric ester. Since all the organic acid-soluble phosphorus of blood is present in the corpuscles, it was necessary, in order to follow the important changes taking place within them, to determine the percentage of corpuscles in each blood sample.

The results are shown in Table I.

Table I. *Changes in phosphorus distribution in blood during ammonium chloride acidosis.*

		In milligrams P per 100 cc. *blood*						In mg. P per 100 cc. *corpuscles*		In mg. P per 100 cc. *blood*	
Day of month	Haematocrit percentage corpuscles	(*a*) Inorganic phosphorus	(*b*) Total acid soluble phosphorus	(*c*) Organic phosphorus (*b* − *a*)	(*d*) Hydrolysable phosphorus	(*e*) Non-hydrolysable phosphorus (*c* − *d*)	(*f*) Percentage hydrolysable phosphorus in whole blood	(*g*) Hydrolysable phosphorus to nearest mg.	(*h*) Non-hydrolysable phosphorus to nearest mg.	(*i*) Lecithin phosphorus	(*j*) phosphorus (*b* + *i*)
29th[1]	46	3·2	27·5	24·3	6·0	18·3	25	13	39	11·2	38·7
30th[2]	48	3·4	24·9	21·5	6·7	14·8	31	14	31	12·1	37·0
31st[3]	51·5	3·9	24·0	20·1	6·6	13·5	33	13	26	12·4	36·4
1st	52·5	3·7	24·4	20·7	7·0	13·7	34	13	26	12·6	37·0
2nd	47·5	2·7	23·1	20·4	6·7	13·7	33	14	29	10·9	34·0
3rd	45·0	2·6	23·3	20·7	5·8	14·9	29	13	33	10·0	33·3
4th	39·5	2·5	22·0	19·5	5·5	14·0	28	14	35	10·1	32·1
5th	39·0	3·0	23·3	20·3	4·9	15·4	24	13	39	10·3	33·6
6th	40·0	3·4	24·9	21·5	4·6	16·9	21	12	42	10·4	35·3
10th[4]	44·0	3·4	27·1	23·7	5·4	18·3	23	12	42	11·3	38·4

[1] Blood sample 10 a.m. 25 g. NH_4Cl eaten between noon and midnight.
[2] Blood sample 9.30 a.m. 25 g. NH_4Cl eaten between noon and midnight.
[3] Blood sample 9.30 a.m. 20 g. NH_4Cl eaten between noon and midnight.
[1, 4] Normal days.

The change in the inorganic phosphate of the blood confirms in general that found in a previous experiment of Haldane, Wigglesworth and Woodrow

[1924]. In the experiment described in the present paper the rise in inorganic phosphorus was more marked and the subsequent fall not quite so severe. In Haldane, Wigglesworth and Woodrow's experiment, sodium phosphate was taken on the fourth day after the last ammonium chloride day and brought the inorganic phosphorus rapidly back to the normal, whereas here the return to the normal level was more leisurely, being first accomplished on the sixth day after the last ammonium chloride day. On this day, although the total acid-soluble phosphorus per 100 cc. blood was not yet back to the normal, the total acid-soluble phosphorus *per* 100 *cc. corpuscles* had already reached its normal value. The discrepancy is due to the fact that the normal percentage of corpuscles had not yet been reached.

The "lecithin" or lipin phosphorus changed in a similar way to the inorganic phosphorus, but it was more clearly connected with changes in the haematocrit value, which is to be expected, since corpuscles contain two and a half times as much lipin phosphorus as the plasma [Bloor, 1916]. The lecithin phosphorus *per* 100 *cc. corpuscles*, calculated from the haematocrit value and the ratio found by Bloor (assuming that this ratio still holds during acidosis) shows only a small variation during the period of the experiment, an initial rise in the first three days being followed by a corresponding fall in the next three days, and a rise again to normal at the end of the period of experiment. The organic acid-soluble phosphorus per 100 cc. blood and per 100 cc. corpuscles shows an immediate fall at the outset, followed by a slow return to normal after several days.

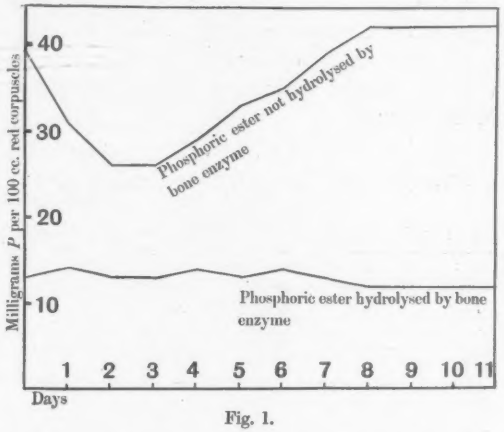
Milligrams P per 100 cc. red corpuscles
40
30
20
10
Phosphoric ester not hydrolysed by bone enzyme
Phosphoric ester hydrolysed by bone enzyme
1 2 3 4 5 6 7 8 9 10 11
Days

Fig. 1.

It is in the partition of this organic acid-soluble phosphorus into enzyme-hydrolysable and non-hydrolysable portions that the most interesting development occurs. Fig. 1 shows the amounts in 100 cc. corpuscles of the enzyme-hydrolysable and non-hydrolysable phosphorus.

It seems clear that the quantity of hydrolysable phosphoric ester in the

corpuscles remains constant during acidosis, and that the total loss of acid-soluble organic phosphorus from the corpuscles is borne by the portion resistant to hydrolysis by the bone enzyme, which portion is evidently one of the reserves to be called upon to make up the loss of phosphorus from the body.

(The urinary excretion of phosphate during this particular experiment is not yet available, but there is no reason to suppose that it will be found markedly different from that in a previous experiment with the same subject, when about the same amount of ammonium chloride was eaten and the maximum excretion of urinary phosphorus was found to occur on the last two days on which this salt was taken. This peak in the excretion curve would thus be associated with the big fall in the non-hydrolysable phosphoric ester in the corpuscles.)

It is unlikely that such a large proportion of the blood phosphorus (18 mg. P per 100 cc. whole blood or six times the amount of inorganic phosphorus) would be carried about by such a highly specialised cell as the red blood corpuscle unless it played some useful part, and the result of this experiment indicates its possible function as a readily available reserve supply of phosphorus, possibly the first reserve called upon, to enable the kidney to excrete acid within the limits of hydrogen ion concentration compatible with the health of this organ.

A further analysis of the figures in Table I reveals the fact that the total loss of phosphorus from the whole of the blood at any stage in the acidosis is very nearly the same as the loss of phosphorus from the enzyme-stable ester fraction of the organic phosphorus in the corpuscles (Table II).

Assuming that the blood volume of the subject was six litres to begin with, and that the corpuscular volume was therefore 46 % of this, i.e. 2·76 litres, and that this corpuscular volume was constant throughout the acidosis, we get the following figures:

Table II.

(After correcting for changes in blood volume.)

	Total P in whole of blood		Non-hydrolysable P in whole of blood		Lecithin P in whole of blood	
Day of month	Total in grams	Excess or deficiency	Total in grams	Excess or deficiency	Total in grams	Excess or deficiency
29th[1]	2·32	+0·0	1·08	+0·0	0·67	+0·0
30th	2·13	−0·19	0·86	−0·22	0·70	+0·03
31st	2·05	−0·27	0·72	−0·36	0·66	−0·01
1st	2·06	−0·26	0·72	−0·36	0·66	−0·01
2nd	1·99	−0·33	0·80	−0·28	0·63	−0·04
3rd	2·04	−0·28	0·91	−0·17	0·61	−0·06
4th	2·05	−0·27	0·96	−0·12	0·71	+0·04
5th	2·32	+0·0	1·08	+0·0	0·73	+0·06
6th	2·37	+0·05	1·16	+0·08	0·72	+0·05
10th[1]	2·39	+0·07	1·16	+0·08	0·71	+0·04

[1] Normal day.

The "lecithin" figures similarly corrected for blood volume are given in the last two columns of Table II. It will be seen that the lecithin rose above

normal during the rapid breaking up of the enzyme-stable phosphoric ester at the beginning of acidosis, and is again above normal during the recovery period. This may possibly mean that this ester both in hydrolysis and synthesis passes through the "lecithin" or ether-soluble stage. Inorganic phosphate may be the next stage in the hydrolysis, as it remains high whilst the ester is being hydrolysed, then falls through normal and reaches its lowest point on the two days when the re-synthesis of the ester, and therefore the demand for phosphorus to make it, is most marked. Perhaps this may be putting more strain on the figures than they can bear, but it is at least a suggestive possibility and connects the sequence of many of the observed changes.

The presence of adenine nucleotide in blood corpuscles has already been mentioned. Quantitatively (taking Jackson's [1924] figures of 15–25 mg. nucleotide per cent.) it accounts for some 2 mg. P per 100 cc. whole blood, or 5 mg. P per 100 cc. corpuscles. The maximum decrease in the non-hydrolysable phosphorus in corpuscles during the acidosis period is 13 mg. P %, so that even were the adenine nucleotide to disappear completely it would only account for the lesser portion of the missing phosphorus. The only known organic phosphorus compound in the corpuscles, therefore, gives no clue to the problem.

That these unknown phosphorus compounds in the corpuscles, which are chemically fairly stable substances, not hydrolysed at room temperatures or at 37° on standing in neutral, alkaline or acid solution, and requiring to be boiled with $N/2$ alkali or acid for many hours before complete hydrolysis is obtained, should be isolated and identified is a matter of some urgency[1]. It is possible that the responsibility for changes in the reducing power of blood filtrates after acid hydrolysis remarked by Cooper and Walker [1921, 1922] and certain anomalies in the relationship of the reducing power to the polarimetric value of blood filtrates such as those observed by Winter and Smith [1922] may find an explanation in the nature of the organic phosphorus compounds in the corpuscles.

Summary.

Marked changes occur in the distribution of phosphorus compounds in human blood during the acidosis which follows the ingestion of ammonium chloride. The significance of these changes is discussed.

The author desires to express his thanks to Mr J. B. S. Haldane for his patience in trying circumstances and for suggestions and criticism.

[1] Dr Robison informs me that he is at present engaged on this work and will shortly publish a paper on the subject.

REFERENCES.

Bloor (1916). *J. Biol. Chem.* **25**, 577.
—— (1918). *J. Biol. Chem.* **36**, 49.
Cooper and Walker (1921). *Biochem. J.* **15**, 415.
—— (1922). *Biochem. J.* **16**, 455.
Haldane, Wigglesworth and Woodrow (1924). *Proc. Roy. Soc. Lond.* B, **96**, 1.
Jackson (1924). *J. Biol. Chem.* **59**, 529.
Kay and Robison (1924). *Biochem. J.* **18**, 755.
Randles and Knudson (1922). *J. Biol. Chem.* **53**, 53.
Robison and Soames (1924). *Biochem. J.* **18**, 740.
Winter and Smith (1922). *J. Physiol.* **57**, 100.

CXLIX. THE ROLE OF PHOSPHATES IN CARBOHYDRATE METABOLISM.

I. THE ACTION OF THE MUSCLE ENZYME ON THE ORGANIC PHOSPHORUS COMPOUNDS OF BLOOD.

II. THE EFFECT OF INSULIN ADMINISTRATION ON THE DISTRIBUTION OF PHOSPHORUS COMPOUNDS IN BLOOD AND MUSCLE.

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(*Received August 1st, 1924.*)

During the fermentation of sugar by yeast juice in presence of phosphates at least two compounds of carbohydrate with phosphoric acid are produced, a hexosediphosphoric ester $C_6H_{10}O_4(PO_4H_2)_2$ and a hexosemonophosphoric ester $C_6H_{11}O_5PO_4H_2$, the position of the acid group in the latter compound being almost certainly different from that of either of the groups in the former.

The interest, which has long surrounded hexosediphosphoric ester and the problem of its rôle in the carbohydrate metabolism of the yeast cell, has been extended by the work of Embden and his collaborators, who have brought forward evidence of its occurrence in muscle and of its probable identity with the precursor of lactic acid. The more recently discovered monophosphoric ester may also have its counterpart in animal physiology. It has been shown [Robison, 1923; Robison and Soames, 1924] that whereas this compound is scarcely acted on by the enzymes of muscle it is rapidly hydrolysed by an enzyme which is secreted in bones and ossifying cartilage and in teeth, and further [Kay and Robison, 1924] that blood contains appreciable amounts of a phosphoric ester which is hydrolysed by this bone enzyme. The light which these facts may throw on the hitherto unexplained process of ossification has been discussed in the papers referred to above, but the exact composition of the blood ester has yet to be determined. The possibility that it consists wholly or in part of a hexosediphosphate was from the first of twofold interest to us. If this could be demonstrated it would provide another clue to the problem of carbohydrate metabolism and a link with Embden's "lactacidogen," but for the suggested scheme of ossification the diphosphoric ester would not furnish a satisfactory substrate, since it is readily hydrolysed by muscle and

non-ossifying cartilage as well as by bone. We therefore planned to investigate the action of both bone and muscle enzymes on the phosphorus compounds in the blood of man and of animals under different conditions of nutrition, using these enzymes as tools for the discovery and quantitative estimation of compounds of the type of hexosemonophosphoric ester and hexosediphosphoric ester respectively. Only the results of the experiments with the bone enzyme, carried out chiefly during 1923, have so far been published [Kay and Robison, 1924] but with most of these samples of blood the action of muscle extracts was simultaneously investigated, adopting the same technique as described for bone extracts. The results were neither so consistent nor so easy to interpret as those obtained with the bone enzyme and they were therefore held back in the hope that further work might enable us to draw more definite conclusions. The difficulty was caused partly by the fact that the amounts of inorganic and of hydrolysable organic phosphates in the muscle extracts were relatively large, while the amounts of hydrolysis observed were small, and partly by the fact that the muscle enzyme is not entirely without action on the monophosphoric ester, although its power is very slight in comparison with that of the bone enzyme.

Although, therefore, some of the results pointed to the presence of a compound of the hexosediphosphate type, it is not possible to state definitely that such a compound is a normal constituent of blood.

It is, however, certain that a large proportion of the phosphoric ester of blood which is hydrolysable by bone is not readily hydrolysed by muscle, and that the requirements of a specific substrate for the scheme of ossification are thus satisfied.

It is true that, so far, no proof has been given that this phosphoric ester is a derivative of a hexose or of any carbohydrate, but the isolation of its barium salt, although still in impure condition [Goodwin and Robison, 1924] provides strong evidence that the ester at least possesses reducing properties and is optically active.

Before proceeding to deal with the second part of this investigation, the effect of insulin administration on the distribution of phosphorus compounds in the body, the results obtained up to this point will be briefly set out.

Action of the muscle enzymes on hexosephosphoric esters and on the phosphoric esters in blood.

From previous experiments [Robison, 1923] it was known that while the bone enzyme hydrolyses hexosemonophosphoric ester and both groups of the diphosphoric ester, the muscle extracts have little or no action on the former but readily hydrolyse at least one of the phosphoric groups of the latter. They appeared to possess less power towards the second group. For the correct interpretation of our results it was necessary to have more precise information on the relative activity of the muscle enzymes with respect to these three ester linkages, under the conditions of our experiments. Table I shows the

rate of hydrolysis of hexosediphosphoric ester by (*a*) 5 % bone extract prepared by macerating the bones of young rats with 20 times their weight of chloroform water, (*b*) 10 % muscle extract prepared by macerating rabbit muscle with 10 times its weight of chloroform water.

The proportion of tissue extract to ester solution and the other experimental details were the same as those given in our previous paper (p. 758), and the concentration of combined phosphorus was of the same order as that of the organic acid-soluble phosphorus in the blood experiments.

Table I. *Hydrolysis of potassium hexosediphosphate at* 37°; p_H 8·4.

	5 % bone extract		10 % muscle extract	
Time, minutes	% of combined P hydrolysed	k $\left(\frac{1}{t_1-t_2}\log\frac{a-x_1}{a-x_2}\right)$	% of combined P hydrolysed	k $\left(\frac{1}{t_1-t_2}\log\frac{a-x_1}{a-x_2}\right)$
100	64	·0044	13	·0006
200	85	·0038	25	·0006
300	92	·0027	33	·0005
600	96	·0010	50	·0005
1400	98	·0004	68	·0003
2800	100	—	82	·0002
4200	—	—	87	·0001

These results show that the second phosphoric group of hexosediphosphoric ester is also hydrolysed by muscle extract although much more slowly than the first, as can be seen from the sharp fall in the value of k (in the last column) after 50 % of the combined P has been hydrolysed. The values of k for the bone enzyme may also be different with respect to the two groups, but this cannot be so clearly deduced from the figures. They are in any case much greater than the values for the muscle enzyme.

The action of muscle extract on the monophosphoric ester is shown in Table II in which the results of a typical blood experiment are set out in detail. This experiment is the same as that for which the results with the bone enzyme were given in our last paper (p. 759). The action of each enzyme was in this case tested in duplicate, at different p_H values. The bone extract at p_H 9·3 and 8·4 gave almost identical figures and only one series was therefore quoted. The muscle extract was tested at p_H 8·4 and 7·3 and here also the results were in close agreement, which proves that the muscle enzyme does not possess the marked high optimum p_H (8·4–9·4) of the bone enzyme. After deducting the amount of the increase in inorganic phosphate due to hydrolysis of phosphoric esters in the muscle extract (OM) it is seen that a very slight hydrolysis of the hexosemonophosphate (MM) was effected by the enzyme in 10 hours. The increase of inorganic phosphate in the case of the blood filtrate (FM) is slightly greater still, from which the presence of a compound of the hexosediphosphate type, in very small amount, might be inferred. The figures are, however, very near to the limit of experimental error, and deductions must be made with caution. Similar results were obtained in most of our experiments—slight hydrolysis, generally a little more than that found in the controls. A summary of these results is given in Table III. Only in one

experiment (no. 17) did we obtain a considerable amount of hydrolysis, quite outside the range of ordinary experimental error, and largely occurring in the first hour. We can find no reason for doubting this result but we have not been able to repeat it. It may be that the particular nutritive condition of the animals was favourable to the accumulation of this ester in the blood. In another experiment (no. 26) on human blood a slow hydrolysis was observed throughout 44 hours—amounting to 1·4 mg. P per 100 g. blood in 6 hours, 2·0 mg. in 20 hours and 3·2 mg. in 44 hours. Almost identical figures were however in this case obtained for a parallel experiment using sodium glycerophosphate with the same muscle extract, so that one must conclude that this blood contained no ester of the diphosphate type.

Table II. *Exp. no.* 33. *Whole blood of an adult rabbit taken from carotid and jugular.*

Hydrolysis by 10 % muscle extract at 37·5°.
Inorganic phosphate (mg. P) in 5 cc. final filtrate—equivalent to 0·5 g. blood.

			Time in hours						Increase after hydrolysis	Net amount of hydrolysis
		p_H	0	1	2	3	5	10		
FM.	Blood filtrate + muscle extract	8·4	·058	·058	·062	·063	·064	·070	·012	·0055
		7·3	·0585	·059	·061	·062	—	·070	·0115	·006
FO.	Blood filtrate + water	8·4	·024	—	—	—	·025	·024	—	—
OM.	Water + muscle extract	8·4	·036	—	—	—	·040	·0425	·0065	—
		7·3	·0365	—	—	—	·0395	·042	·0055	—
MM.	Sodium hexosemonophosphate + muscle extract	8·4	·0375	·038	—	—	·042	·046	·0085	·002
		7·3	·0375	·038	—	·039	·043	·047	·0095	·004
DM.	Sodium hexosediphosphate + muscle extract	8·4	·045	·057	·062	—	·083	·103	·058	·0515
		7·3	·0475	·058	·063	0·69	·082	·102	·0545	·049

	Mg. P per 100 g. blood
Total acid soluble phosphorus	= 31·0
Inorganic phosphate	= 4·8
Phosphoric esters	= 26·2
of which (*a*) esters hydrolysed by bone enzyme	= 6·5
(*b*) esters hydrolysed by muscle enzyme	= 1·2
Hexosemonophosphoric ester hydrolysed under similar conditions by muscle enzyme	= 0·6 (Total organic P = 21·6)
Hexosediphosphoric ester hydrolysed under similar conditions by muscle enzyme	= 10·0 (Total organic P = 23·0)

The effect of insulin administration on the distribution of phosphorus compounds in blood and muscle.

The evidence at this stage appeared to show that a hexosediphosphoric ester may occur in blood, but that under the conditions of carbohydrate metabolism usual in our experiments the amounts are too small to be satisfactorily demonstrated by our method. By disturbing the normal carbohydrate metabolism the necessary conditions might be obtained whereby the amount of this substance could be increased.

The possibility presented itself of realising these conditions by the use of insulin, particularly in view of the recent work which had shown that the

Table III.

(For other data of these experiments see Kay and Robison [1924, Table I[1]].

				Mg. P per 100 g. blood			
				Hydrolysis by muscle enzyme			
No. of Exp.	Animal	Age	Type of blood	Strength of muscle extract %	Duration of experiment hrs.	Amount hydrolysed	Hydrolysed by bone enzyme
9	Cockerel	6 months	Mixed	10	18	1·0	3·2
8	Rats	3 „	„	10	18	2·1	7·4
11	„	3 „	„	2½	22	2·4	7·4
12	„	2½ „	„	5	26	1·2	4·7
	„	9 „	„	5	26	2·2	5·4
17	Rabbit	3½ „	„	10	1	4·6	—
					24	7·8	9·9
	„	18 „	„	10	1	2·5	—
					24	3·9	6·5
33	„	adult	„	10	10	1·2	6·5
40–49	„	young adults	average of 10	10	18	2·8	8·7
27	„	adult	venous	10	3½	0·6	8·5
	„	„	arterial	10	3½	0·7	8·7
28	Child ♀	10½ years	venous	10	8	1·7	5·7
	„ ♂	11 „					
26	Man	adult	„	10	6	1·4	6·0

[1] Correction. In this table (p. 760) line 13 should read: "Exps. 40–49. Rabbits—Young adults —Average of 10—Total acid-soluble, 34·5; Inorganic, 5·7; Organic, 28·8; Hydrolysed by bone, 8·7; Not hydrolysed, 20·1; Percentage of organic P hydrolysed by bone, 30."

inorganic phosphate decreases as well as the reducing substance. The decrease is greater than can be explained solely by the increase in blood volume which occurs after insulin administration [Haldane, Kay and Smith, 1924]. Thus in Table IV the first line A shows the figures obtained in some experiments of our own on seven young adult rabbits; line B shows those obtained by Wigglesworth, Woodrow, Winter and Smith [1923] on adult rabbits (average of 6 taken from their protocols); line C shows the serum phosphate results of Harrop and Benedict [1924].

Table IV.

	Inorganic phosphate (mg. P) per 100 cc. blood		
	Before insulin	After insulin	Percentage diminution
A	6·0	4·5	25
B	5·6	3·8	32
C	6·4	3·9	38
Average	6·0	4·1	32

Under the conditions of our experiments (line A) the blood volume increases some 15 %, so that the diminution in inorganic P is a real one, and indicates that inorganic phosphate is actively taken out of the plasma by the body cells after insulin administration. In the tissues, which include the red blood corpuscles, there is a "phosphate vacuum" no less than a "glucose vacuum."

By taking into account these blood volume changes we can examine the hexosephosphate hypothesis on a quantitative basis.

If we take the original blood volume of the 2 kg. rabbit as 110 cc. the increase in blood volume between the administration of insulin and the onset of convulsions (15 %) brings the final blood volume to 126 cc. Taking 6·6 mg. P as the total original inorganic P in 110 cc. blood, and 5·1 as the total inorganic P in 126 cc. blood at the convulsive stage, we see that 1·5 mg. P disappears into the tissues. Assuming that a hexosemonophosphate is synthesised and is taken out of the blood, this amount of P would account for 9 mg. glucose or for 4·5 mg. if a hexosediphosphate were formed. The reducing power of these esters is respectively about 66 % and 33 % of their sugar equivalent so that if they remained in the blood the apparent loss of glucose would be correspondingly less.

Actually the amount of sugar which is taken out of the blood is much greater than this, being very approximately the difference between [0·11 % of 110] g. and [0·045 % of 126] g. Assuming for the time being that there is no mobilisation of sugar from glycogen or other reserves in the other tissues, this gives 65 mg. glucose as the least amount leaving the blood. It is evident that the amount of inorganic phosphate leaving the blood is only sufficient to account, even on the hexosemonophosphate basis, for a maximum of some 14 % of the glucose which is simultaneously being taken out of the blood stream. If an intermediate hexosephosphoric acid complex is to account for the whole of this glucose, phosphate must be provided by some other tissue of the body, in which case an increase in organic at the expense of inorganic phosphate (possibly in the same tissue) may be found, or a change in the state of combination of part of the organic phosphate originally present. Some evidence as to the truth or otherwise of this hypothesis is given below.

Possible changes in the blood.

Although the drop in inorganic phosphate in the blood was insufficient to account for more than 14 % of the fall in glucose, it was possible that the phosphoric esters might have increased in amount. As these are present, both before and after insulin, almost entirely in the corpuscles, the relative amounts of corpuscles and plasma which are altered by the administration of insulin, must be known if the results are to have any value.

The distribution of phosphorus compounds in blood after insulin was investigated by means of the bone and muscle enzymes as already described. In all the determinations shown below (except those of "lecithin" P) two strictly comparable animals, usually from the same litter, were used, the first for the control figures being given saline and treated to the same operative technique as the second animal which received the same volume of insulin solution. The insulin animal was killed by bleeding either just before or actually at the onset of convulsions and the mixed arterial and venous blood used for our determinations. The control was treated in the same way, in some cases just before, in others shortly after the death of the insulin animal.

The results of a series of experiments are summarised as follows:

Table V[1].

Type of phosphorus compound	Control mg. P per 100 cc.	After insulin mg. P per 100 cc.	Decrease %	Remarks
1. Lecithin	10·8	9·1	16	Average of 7 exps.
2. Total acid-soluble	36·3	34·6	5	Average of 11 exps.
3. Inorganic	6·0	4·5	25	,, ,,
4. Organic	30·3	30·1	1	,, ,,
5. Hydrolysable (bone enzyme)	9·2	9·8	Increase 6	,, ,,
6. Hydrolysable (muscle enzyme)	2·9	2·7	Decrease 6	Average of 9 exps.

In considering the figures for the percentage decrease in the concentration of the various phosphorus compounds we must bear in mind that the blood volume is increased by about 15 % after insulin, and the corpuscles, which contain practically the whole of the organic acid-soluble phosphorus compounds, are diluted by this amount.

We may therefore draw the following tentative conclusions:

(1) The decrease in the lecithin P (16 %) is due entirely to the increase in the blood volume, i.e. no lecithin P enters the blood with the infiltrating fluid (or else the amounts of lecithin entering and leaving the blood during the experimental period balance).

(2) The apparent decrease in total acid-soluble P (5 %) corresponds with an increase in absolute amount equivalent to about 9 %, or 3·5 mg. P per 100 cc. of the original blood volume. This extra phosphate as well as that required to compensate for losses through the kidneys, must be drawn from the other tissues and may be partly derived from the breakdown of phosphoric esters or in the last resort from bone.

(3) The inorganic phosphate suffers a considerable decrease both in percentage (25 %) and in absolute amount (about 0·8 mg. P per 100 cc. of the original blood volume).

(4) The organic acid-soluble P thus undergoes an increase in absolute amount equivalent to nearly 14 % or 4·3 mg. P per 100 cc. of the original blood volume.

(5) The esters hydrolysable by the bone enzyme are also increased but the absolute amount of this increase (2·1 mg. P per 100 cc.) is less than that of the total organic acid-soluble P.

(6) It would seem most probable therefore that a synthesis of organic phosphorus compounds from the inorganic phosphates takes place in the corpuscles after insulin, and it is reasonable to assume that in this synthesis the sugar which simultaneously disappears is also concerned. This does not necessarily imply that the phosphoric ester thus formed is a simple hexose derivative. It may be of a more complex nature—possibly a nucleotide [cf. Jackson, 1923].

[1] Our previous results have been given as mg. P per 100 g. For the insulin experiments the blood was usually measured and the results are given per 100 cc. in order to simplify the correction for blood volume changes.

(7) On the basis of one phosphoric group per molecule of glucose 4·3 mg. P would account for 25 mg. glucose or 39 % of the sugar disappearing from the blood.

In such an investigation as this in which different animals must necessarily serve as controls it is obviously wise to draw conclusions only from the averages of as large a number of individual experiments as possible. The above conclusions may however be illustrated by taking the results of a single experiment on three young rabbits (12 weeks old) from the same litter.

A. Wt 755 g. Control.
B. Wt 735 g. Received 1·5 cc. insulin. Killed 2 hours later.
C. Wt 860 g. „ „ „ 3 „

All three animals fasted during 24 hours before the experiment. Convulsions had not set in at death of either animal. The results obtained with the whole blood and plasma are shown in Table VI.

Table VI. *Whole blood.*

	A (control) %	B (insulin) %	C (insulin) %
Hb before injection	—	69	72
Hb at death	69	56	58
Sugar	·138	·048	·048
	Mg. P per 100 cc.		
Inorganic PO_4	8·2	6·5	6·6
Total acid-soluble P	38·0	34·4 [42·2]*	36·7 [45·6]
Organic acid-soluble P	29·8	27·9 [34·4]	30·1 [37·5]
Ester hydrolysable by bone	6·5	6·4 [7·9]	5·0 [6·2]
Increase in absolute amounts of organic P	—	[4·6]	[7·7]
Glucose equivalent to above (1 mol. glucose : 1 mol. H_3PO_4)	—	27 mg. = 33 % of sugar disappearing	45 = 54·7 % of sugar disappearing
Plasma.			
Inorganic PO_4	10·2	6·7	6·7
Total acid-soluble P	10·2	6·7	6·7
Organic acid-soluble P	0	0	0

* The figures in brackets show the amounts of P corrected for blood dilution as shown by the change in Hb $\left(\text{i.e.} \times \frac{\text{Hb before injection}}{\text{Hb at death}}\right)$.

Some further light is thrown on this question by the results of recent experiments of Lawaczeck [1924], Martland and Robison [1924], Martland, Hansman and Robison [1924], who have shown that blood contains an enzyme which can effect both the hydrolysis and synthesis of phosphoric esters *in vitro*, the direction of the reaction depending on the conditions as regards p_H, etc.

It is likely that the blood corpuscles play a more important rôle in sugar metabolism than is usually attributed to them. When we consider that in the yeast cell a relatively small amount of phosphate through a continuous

cycle of reactions, effects the metabolism of large amounts of carbohydrate the significance of the figures in Tables V and VI becomes emphasised. It must be remembered also that although the greater part of the phosphoric esters in the corpuscles are apparently not simple hexose derivatives and are non-reducing (cf. Goodwin and Robison) it is quite possible and even likely that a carbohydrate nucleus is present in their molecules, as for example in the nucleotides whose occurrence in blood has been confirmed by Jackson [1924]. It is an interesting calculation that if each atom of organic acid-soluble P in the corpuscles is associated with one molecule of sugar there is in 100 cc. of blood no less than 160–200 mg. of hidden sugar thus bound up, i.e. considerably more than there is reducing sugar in normal blood.

Changes in the muscle.

The changes in the phosphorus partition in the muscle were examined at the same time, and were found to be as definite as in the blood. Considerable increases were found in the "lactacidogen" phosphoric acid of muscle after insulin whilst the inorganic phosphate and the total acid-soluble phosphorus remained practically unchanged.

The method employed was as follows. Two comparable rabbits usually from the same litter were given the same diet for a preliminary period of several days. They were then starved for about 18 hours and one was given an injection of sufficient insulin to produce convulsions in about 3 hours, the other being given the corresponding quantity of saline. The two animals were then watched until signs of the approach of convulsions were noticed in the insulin animal, when it was killed by bleeding (without anaesthetic). The gastrocnemius and soleus muscles free from connective tissue were immediately removed, weighed and divided exactly into halves, one half being immediately macerated with an ice-cold 2·5 % solution of trichloroacetic acid and sand in an ice-cold mortar. The time from the death of the animal to the complete maceration of the muscle was usually from 3 to 5 minutes. The volume of the mixture (M.T.) was made up to 40 cc. per g. of muscle. The other half of the muscle was ground up with sand and ice-cold water and made up to the same volume (M.A.). In some of the experiments a 0·25 % solution of $NaHCO_3$ was used instead of distilled water. When this was not done the mixture was neutralised with NaOH (to phenolphthalein) either immediately or after 3 hours at 38°.

The control animal was treated in exactly the same way within a short time of the death of the insulin animal, and the autolysis mixtures (M.A.) from both were then kept at 38° in presence of chloroform.

The trichloroacetic acid mixtures (M.T.), after 3 hours at room temperature with occasional shaking, were filtered and neutralised, and the inorganic phosphate then estimated by the Briggs modification of the Bell-Doisy method. They were also treated with the bone and muscle enzyme preparations according to the technique already described for blood filtrates, and the amount of organic phosphate hydrolysed was determined after 18 hours at 38° and p_H 8·4.

The earlier experiments showed somewhat greater amounts of hydrolysis in the muscle extracts from the animals which had received insulin than in those from the controls, but later experiments have rendered this less certain, and the average of eight experiments (Table VII) shows little difference in the amounts of hydrolysis under parallel conditions either by the bone or the muscle enzyme. In the case of the bone enzyme the hydrolysis at this stage was practically complete, only a very slight further increase being obtained after another 18 hours at 38°.

Table VII.

	Amount of hydrolysable acid-sol. P in mg. per 100 g. muscle		
	Control	After insulin	
Bone enzyme, 18 hours	74	76	Average of 8 exps.
Muscle enzyme, 18 „	11	12	Average of 6 exps.
„ „ 36 „	19	18	„ „

With the muscle enzyme, however, slow hydrolysis was still proceeding at the end of 36 hours, and the results are only of significance in suggesting that the greater portion of the phosphoric ester extracted from muscle by trichloroacetic acid is not identical with the hexosediphosphate obtained by the action of yeast juice on sugar, since this ester would have been much more rapidly hydrolysed by the muscle extract used.

Although the use of the bone and muscle enzymes on trichloroacetic extracts yielded these equivocal results, the data obtained by the autolysis of the other halves of the muscle, macerated with water or $NaHCO_3$ solution, gave more definite evidence of changes in the nature and amounts of the phosphoric esters present at the convulsive stage following insulin injection.

Samples were taken from the muscle autolysis mixtures (M.A.) at intervals, usually after 3 hours, 8 hours, and at 60 hours, when the inorganic phosphate and total phosphorus were determined in aliquot portions of the mixture after precipitation of proteins with trichloroacetic acid and filtration.

As a result of ten experiments the following average figures were obtained:

Table VIII.

	Inorganic phosphate (mg. P) per 100 g. muscle					Total P in solution at 60 hrs.
Duration of autolysis (hrs.)...	0	3	8	12	60	
Control animal	95	140	144	*	172	194
Increase		45	49		77	
Insulin animal	92	161	164		179	196
Increase		69	73		87	
Difference (Insulin-Control)	−3	+21	+20	+16	+7	+2

* A few determinations at 12 hrs. gave an average difference of 16 mg.

It will be seen from these figures that:

(1) The amount of inorganic phosphate in rabbit muscle is not appreciably changed after the administration of insulin (but before the onset of convulsions).

(2) The amount of total acid-soluble phosphorus (present after 60 hours' autolysis) is also not changed by insulin.

(3) The amount of combined phosphoric acid which is rapidly set free during autolysis is considerably increased (nearly 50 %) after insulin.

(4) After more prolonged autolysis of the macerated muscle, the difference between the amounts of inorganic phosphate set free in both series (insulin and control) becomes less, and approaches zero after 60 hours at 37°.

During the long-continued autolysis, phosphoric acid may be liberated by the slow action of other enzymes on a variety of organic phosphorus compounds so that the chief significance of our results seems to us to lie in the marked difference between the amounts of phosphate liberated during the first few hours (the "lactacidogen" P).

The increase in rapidity of hydrolysis of the organic phosphate in the macerated muscle aroused the suspicion that insulin might possibly act as a co-enzyme to the phosphoric esterase of muscle, assisting in the synthesis of the ester *in vivo* and in its hydrolysis under the conditions of our experiments *in vitro*. This attractive hypothesis was tested in two experiments with potassium hexosediphosphate solution and muscle enzyme (derived from normal muscle) but the results gave not the slightest indication that insulin had any co-enzymic action. The possibility that the phosphoric esterase of muscle might be otherwise more active after the injection of insulin was also investigated, but no appreciable difference was found, using potassium hexosediphosphate as substrate.

According to Embden the lactacidogen is either hexosediphosphate or a very closely related compound. The rise in rapidly hydrolysable phosphate we may interpret as indicating that the lactacidogen is increased in muscle after the administration of insulin. The source of the phosphorus for the increased amount of this substance appears to be not the blood phosphate but the other phosphoric acid complexes (possibly nucleic acid) in the muscle which are much less readily autolysed than is lactacidogen by the muscle enzymes.

We have no evidence that the carbohydrate portion of the lactacidogen was not also present in the muscle before the administration of insulin, e.g. such a compound of sugar and phosphoric acid might conceivably be formed from a nucleotide by the removal of the purine or pyrimidine group. If, however, the sugar for the increased synthesis of this substance is taken up from the blood the rise in lactacidogen would account quantitatively for much more than the whole of the sugar which thus disappears.

If we assume that the muscles of a 2 kg. rabbit together weigh 800 g. and that the percentage increase of lactacidogen is the same throughout, an increase of 8×21 mg. combined P is found in the muscles, using the 3-hour figure from Table VIII.

If this phosphate is combined with sugar in the molecular ratio of 1 : 1 (hexosemonophosphate) this would account for 980 mg. glucose, or if the

ratio is 2 : 1 (hexosediphosphate) for 490 mg. The actual amount of sugar which disappears from the blood of a 2 kg. rabbit, previously calculated, is only 65 mg. but this does not allow for mobilisation of sugar from glycogen or other reserves[1].

The evidence afforded by our experiments is insufficient to enable us to come to any definite conclusion as to the correctness of this hypothesis but the recent experiments of Dale [1924], Dudley and Marrian [1923] and of Winter and Smith [1924] and others have shown that the sugar which disappears from the blood is neither oxidised nor excreted nor found as increased glycogen nor as fat in the tissues, even when isolated perfused organs are used, but is stored in some unknown form.

We think that the results shown in this paper strengthen the probability that in the mechanism of this storage the phosphoric group plays an important part and that the stored compound itself may prove to be a phosphoric ester.

While these experiments were in progress a short preliminary notice was published by Audova and Wagner [1924, 1, 2] outlining the results of five experiments done in Lemberg in which similar findings had been made as far as the readily autolysable organic phosphoric acid complex in muscle was concerned. The increase in lactacidogen P was of the same order as in our experiments. Their technique was different from our own, and their phosphate determinations seem uniformly a little higher.

Approaching the subject from a different side Harrop and Benedict [1924] have still more recently found similar results to follow insulin injection, though using a technique which might be expected to cause serious changes in the blood phosphate as the effect of shock [cf. Martland and Robison, 1924].

(A decimal point appears to have gone astray in all the muscle phosphate figures given in their Table III.)

Summary.

The phosphoric esters of blood have been investigated by means of the muscle enzyme which hydrolyses hexosediphosphoric esters but has scarcely any action on hexosemonophosphoric ester. While the results indicate that an ester of the hexosediphosphoric type may occur in blood the evidence on this point is not entirely conclusive.

It is, however, certain that the greater part of the organic phosphate which is hydrolysable by the bone enzyme is not hydrolysed by muscle. In this respect the ester therefore satisfies the important condition of a specific substrate in the scheme of ossification.

Changes in the distribution of phosphorus compounds in blood and in muscle after the administration of insulin have also been investigated.

[1] The effect of blood volume on these quantitative relationships is small. If the volume of blood increases from 110 to 126 cc. and if this fluid comes entirely (though this is most unlikely) from the muscular tissue the maximum increase in the concentration of indiffusible substances in the muscle would not exceed 2 %, which is within the experimental errors of the method.

From the average results of 11 experiments on rabbits and taking into account the increase in the blood volume, evidence is produced that after the administration of insulin, organic phosphoric esters are synthesised in the corpuscles at the expense of the inorganic phosphate originally present in the blood plus additional phosphate drawn from the other tissues.

These esters are chiefly of the type not hydrolysed by the bone enzyme but it is none the less possible and is even probable that sugar or a sugar derivative enters into their molecule.

On the basis of one molecule glucose per atom of P the phosphoric acid thus esterified would account for nearly 40 % of the sugar simultaneously disappearing from the blood.

No changes were found in the amount of inorganic phosphate or total acid-soluble phosphorus in muscle, following insulin administration, but there was a marked increase (50 %) in the amount of phosphoric esters ("lactacidogen" P) hydrolysed during 3 hours' autolysis of the macerated muscle at 38°.

The phosphate for this increased "lactacidogen" is apparently drawn from the other phosphorus compounds in the muscle, but we have no evidence as to the origin of the carbohydrate portion of its molecule.

Assuming that lactacidogen is a hexosediphosphate the increased amount found in muscle after insulin would, if the hexose were derived from the blood sugar, account for more than seven times the amount of glucose which disappears from the blood.

REFERENCES.

Audova and Wagner (1924, 1). *Klin. Wochensch.* **3**, No. 6.
—— (1924, 2). *Compt. Rend. Soc. Biol.* **90**, 308.
Dale (1924). Communication to Physiological Soc., Feb. 16.
Dudley and Marrian (1923). *Biochem. J.* **17**, 435.
Goodwin and Robison (1924). *Biochem. J.* **18**, 1161.
Haldane, Kay and Smith (1924). *J. Physiol.* In press.
Harrop and Benedict (1924). *J. Biol. Chem.* **59**, 683.
Jackson (1923). *J. Biol. Chem.* **57**, 121.
—— (1924). *J. Biol. Chem.* **59**, 529.
Kay and Robison (1924). *Biochem. J.* **18**, 755.
Lawaczeck (1924). *Biochem. Z.* **145**, 351.
Martland and Robison (1924). *Biochem. J.* **18**, 765.
Martland, Hansman and Robison (1924). *Biochem. J.* **18**, 1152.
Robison (1923). *Biochem. J.* **17**, 286.
Robison and Soames (1924). *Biochem. J.* **18**, 740.
Wigglesworth, Woodrow, Winter and Smith (1923). *J. Physiol.* **57**, 447.
Winter and Smith (1924). *J. Physiol.* **57**, Proc. xxxi.

CL. THE PHOSPHORIC-ESTERASE OF BLOOD.

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In a previous communication [Martland and Robison, 1924] it was shown that after blood has been laked with distilled water a rapid increase in the amount of inorganic phosphate occurs even at room temperature. The change is still more rapid at 37° but is entirely inhibited when the blood is laked with 1 % trichloroacetic acid, which indicates that it is due to the enzymic hydrolysis of phosphoric esters in the blood. The possibility that in these esters the phosphoric acid is combined with sugar or a sugar derivative suggested that this enzyme (both in its hydrolytic and synthetic function) might play a part in carbohydrate metabolism. We have therefore continued the investigation of its properties and have also sought for any changes in the blood sugar which might be correlated with the action of this enzyme on the organic phosphates.

In the first place our plan was to find the optimum conditions for the hydrolysis and if possible also for synthesis of the blood phosphoric esters by the enzyme, the location of the enzyme (whether in plasma or corpuscles) and the type of phosphoric ester which it can hydrolyse. It was also of interest to find whether the organic phosphate of blood, which is hydrolysed by this enzyme, is the same as that which is acted on by bone and whether any increase occurs in the reducing sugar of the blood as the result of the hydrolysis. The presence of this phosphoric esterase in blood has been also noticed by Lawaczeck [1924] whose paper did not come to our notice until most of our experiments were completed. With regard to certain of the conditions affecting these enzymic reactions his results are similar to those which we have obtained using different methods. He also gives interesting data with regard to the influence of different ions and of CO_2 on the action of the enzyme. Some of these experiments we have repeated and confirmed. His paper does not deal with the question of glycolysis.

Hydrolysis of the phosphoric esters in laked blood.

The course of the hydrolysis in laked blood was studied over periods of several days in order to find whether the whole of the acid-soluble phosphoric esters were decomposed by the blood enzymes.

A quantity of human venous blood (containing oxalate) was laked with distilled water and the volume made up to five times that of the blood. 5 cc. of the laked blood and 0·1 cc. chloroform were pipetted into a number of test tubes, which were closed with rubber stoppers and kept at 38°. At intervals the amount of hydrolysis was measured by adding 5 cc. of a solution of 5 % trichloroacetic acid to one of the tubes to precipitate the proteins and estimating the inorganic phosphate (by Briggs' method) in the filtrate. The total P in this filtrate was also estimated in order to find whether any acid-soluble P (glycerophosphate) was being produced from the lipins by enzymic removal of the fatty acid groups.

Another portion of the same blood was centrifuged and the plasma and corpuscles, each diluted five times with distilled water, were examined in the same way as the whole blood. In each case the initial amount of phosphate was estimated separately by laking 1 cc. whole blood etc. in presence of trichloroacetic acid to prevent enzyme action. The results are shown in Table I.

Table I. *Hydrolysis of phosphoric esters in whole blood, corpuscles and plasma at* 38°.

	mg. P per 100 cc.								
Time in hrs....	0	3	6	22	30	46	72	96	120
Whole blood:									
Inorganic phosphate	3·02	8·05	11·7	22·0	22·2	26·1	—	27·7	28·2
Total acid-soluble P	—	30·1	29·2	30·4	—	30·7	30·7	29·8	30·2
Plasma:									
Inorganic phosphate	3·00	—	—	3·17	—	3·38	—	—	—
Total acid-soluble P	3·50	3·52	—	—	—	3·57	—	—	—
Corpuscles:									
Inorganic phosphate	2·97	10·8	15·7	25·6	28·4	37·6	46·1	49·3	—
Total acid-soluble P	52·2	52·3	54·3(?)	52·4	52·6	52·1	52·1	52·6	—

The constancy of the figures for total acid-soluble P (within the experimental error) is evidence of the absence of any lecithinase from blood—or at least of its inactivity under these conditions. The inorganic phosphate in the laked whole blood and in laked corpuscles increases rapidly at first and then more slowly, until after 4 days it amounts to 93 % of the organic acid-soluble P in both cases. Hydrolysis was at this stage proceeding with extreme slowness.

The small amount of organic acid-soluble P in the plasma is also hydrolysed with extreme slowness, suggesting that it is of the same type as that forming the last 7 % of the phosphoric esters in the corpuscles or else that the enzyme is absent from the plasma. The former alternative is probably the correct one.

Between 22 and 30 hours there appears to be a temporary slowing down in the rate of hydrolysis. We have also noticed this in other experiments, but do not know what significance it may possess.

The optimum p_H *of the enzyme.*

In order to obtain some idea of the optimum p_H for this phosphoric esterase we prepared a series of tubes containing 5 cc. of laked whole blood (1–4 H_2O), and to each added one or more drops of 0·5 *N* H_2SO_4, or NaOH, or H_2O. The

amount of hydrolysis was measured after 2 hours at 38°. The values of p_H shown in Table II are only approximate, but it is obvious that the rate of hydrolysis is greatest at a p_H slightly on the acid side of the normal blood reaction and close to the neutral point. The phosphoric esterase of the blood is thus markedly different from that of the bone, which has an optimum p_H of 8·4–9·4 [Robison and Soames, 1924].

Table II.

Approximate p_H	Amount of hydrolysis after 2 hrs. at 38° mg. P per 100 cc. blood
5·8	1·4
6·2	2·3
6·4	4·0
7·0	6·3
7·4	4·8
7·8	4·9
8·2	1·8
8·6	1·4

The location of the enzyme.

The absence of any rapid hydrolysis in unlaked blood and its onset after rupture of the corpuscles by laking suggested that the enzyme might occur only in the plasma. Against this was to be placed the fact that in the experiment (Table I) the rate of hydrolysis in the laked corpuscles was even higher than that in the whole blood, although the amount of adherent plasma pipetted with the corpuscles must have been relatively small. Experiments were carried out to obtain more information on this point. Since the plasma contains only negligible amounts of the esters it was necessary to provide additional substrate in the form of a neutralised trichloroacetic acid filtrate from the whole blood. The experiment was made with mixed arterial and venous blood (containing oxalate) from a rabbit, part being used for the preparation of the protein-free filtrate (F) and part being centrifuged for the preparation of the laked corpuscles (C) and diluted plasma (P) (both 1 in 5) as in the first experiment. The corpuscles were not washed.

A number of tubes was prepared, each containing:

2 cc. of C or P (equivalent to 0·4 cc. corpuscles or plasma);

{ 5 cc. of F (equivalent to 0·455 cc. whole blood); CF, PF,
{ or 5 cc. H_2O; CO, PO;

0·1 cc. chloroform.

They were stoppered and kept at 38°, tubes from each series being removed at intervals for estimation of inorganic phosphate.

The results, shown in Table III, are expressed as mg. P per 100 cc. corpuscles and plasma respectively. On this basis the organic P in 5 cc. of the blood filtrate, F, represented an additional 34·7 mg. P, but even this did not bring the amount of substrate in PF up to half of that present in the corpuscles alone (CO).

The rate of hydrolysis is almost identical in CO and CF as would be expected

from the large excess of substrate present in both. The fact that in both cases the rate drops considerably and equally after 24 hours would seem to indicate failure of the enzyme rather than the completion of hydrolysis of one of two or more substrates.

In PF the initial rate of hydrolysis was much slower than with the corpuscles but after 72 hours the difference in the rates had almost disappeared. One must conclude therefore that the enzyme is present in both plasma and corpuscles or else that, being in colloidal solution in the plasma it becomes adsorbed on to the surface of the corpuscles either in the body or during centrifugalisation.

Table III.

	Total organic acid soluble mg. P	Amount of hydrolysis expressed as inorganic phosphate mg. P per 100 cc. corpuscles or plasma			
No. of hours...	0	24	48	72	144
CO. Laked corpuscles + water	83·7	25·0	29·4	33·1	40·5
CF. Laked corpuscles + blood filtrate	118·4	25·2	31·2	34·9	41·7
PO. Diluted plasma + water	0·7	0·4	0·6	0·7	0·8
PF. Diluted plasma + blood filtrate	35·4	6·5	10·3	11·8	16·7

Action of the blood enzyme on various phosphoric esters.

The action of the enzyme was tested on a number of naturally occurring phosphoric esters. Human blood (venous) was laked by dilution with distilled water (1 in 5) and 2 cc. of this fluid was at once pipetted into a number of tubes each containing 1 cc. of the ester solution + 0·1 cc. chloroform. These solutions were all brought to p_H 7·0. Into one tube of each series 1 cc. of a 10 % solution of trichloroacetic acid was pipetted before adding the diluted blood, and these tubes provided the figures for zero time. The remainder were kept at 38° for the specified time, after which 1 cc. of the trichloroacetic solution was added. Control tubes containing 2 cc. water + 1 cc. ester solution were also kept at 38° for the same time. The amount of inorganic phosphate in 1 cc. of the filtrates after subtracting the amount at zero time, and allowing for any chemical hydrolysis as shown by the controls, gave the amount of ester hydrolysed by 0·1 cc. blood (0·5 cc. diluted blood).

Table IV. *Hydrolysis of various phosphoric esters by the blood enzyme.*

Ester	Total organic P mg.	Net amount of hydrolysis (mg. P) effected by 100 cc. human blood	
		20 hours	40 hours
Sodium glycerophosphate	780	—	14·2
Potassium hexosemonophosphate	780	11·4	15·4
Potassium hexosediphosphate	1160	144	303
Sodium nucleate	780	0	0
Sodium caseinogenate	780	0	0
Phytin	780	0	0
		Rabbit's blood	
		24 hours	48 hours
Mixed nucleotides from yeast nucleic acid	310	34	54

The results shown in Table IV have been multiplied by 1000 and therefore give the amounts hydrolysed by 100 cc. blood. In the case of sodium nucleate difficulty was caused in estimating the phosphate by the formation of a precipitate and the results are a little doubtful. The action of the enzyme on nucleotides was tested by a separate experiment using rabbit's blood. The mixture of nucleotides was prepared from yeast nucleic acid by the method of Jones and Perkins [1923]. In this experiment the reducing sugar was also estimated, but no evidence of the liberation of any reducing compound was found.

It is seen that the phosphoric esterase of the blood resembles that of muscle in its ability to hydrolyse hexosediphosphoric ester, and in its relative inactivity towards hexosemonophosphoric and glycerophosphoric esters, which were hydrolysed at only 1/20 the rate of the diphosphate and only 1/300 the rate of hydrolysis of the monophosphate by the bone enzyme (from the same weight of bone). It is differentiated both from muscle and bone enzymes by its ability to hydrolyse the unknown phosphoric esters of the blood. It has no action on caseinogen, phytin or nucleic acid but is able to hydrolyse the individual nucleotides.

Comparison of the effect of the blood and bone enzymes on the phosphoric esters of the blood.

It was of interest to find what action the phosphoric esterase of the blood has on that portion of the organic acid-soluble compounds which are hydrolysed by bone extracts. This was done by allowing laked whole blood to autolyse for a certain time and then adding trichloroacetic acid to destroy the enzyme and precipitate the proteins. The neutralised filtrate was examined with bone enzyme by the method described by Kay and Robison [1924, 1]. The protein-free filtrate from another sample of the original blood—laked with trichloroacetic acid—was also treated with bone enzyme in the same way. Table V gives the results of two experiments on human blood (from different subjects), one after 20 hours' and the other after 140 hours' autolysis.

Table V. *Hydrolysis by bone extract of laked human blood before and after autolysis.*

	mg. P per 100 cc.			
	Inorganic	Organic acid-soluble	Hydrolysed by bone	% of organic P hydrolysed by bone
I. Blood as drawn (venous)	2·64	24·0	6·6	27
Do. after 20 hrs. autolysis at 38° (laked)	10·7	15·7	3·4	22
II. Blood as drawn (venous)	3·0	23·0	6·3	27
Do. after 140 hrs. autolysis at 38° (laked)	22·7	3·5	2·6	74

Apparently a portion of the esters hydrolysable by bone is also hydrolysed by the blood enzyme during the early stages of autolysis, but the remaining

portion is very resistant to the action of the latter, and constitutes that residue of organic phosphorus compounds which was found in all experiments after prolonged autolysis. This seems to confirm the results obtained by Kay and Robison [1924, 2] using the muscle enzyme, and to suggest the presence of compounds of both the hexosemonophosphate and hexosediphosphate type.

Changes in the sugar content of the blood during hydrolysis of the phosphoric ester.

The possibility that the hydrolysis of the phosphoric esters might be accompanied by an increase in the amount of reducing sugar in the blood was investigated in a number of experiments, the sugar being estimated in the neutralised trichloroacetic acid filtrates by the method of Hagedorn and Jensen [1923].

Some of the results are shown in Table VI.

Table VI. *Changes in the amount of reducing sugar during autolysis of laked blood.*

Exp. 1. Human blood laked with distilled water (1 in 5).

Time (hrs.)...	0	20	40	64	112	160	184
Inorganic PO_4 (mg. P)	2·64	11·6	16·3	16·3	18·0	20·0	22·0
Sugar (mg.)	118	116	116	116	120	128	150

Exp. 2. Human blood laked with distilled water (1 in 5).

Time (hrs.)...	0	24	44	92	116	142	166
Inorganic PO_4 (mg. P)	3·0	10·6	13·9	17·1	17·8	19·2	20·8
Sugar (mg.)	128	128	150	173	—	173	173

Exp. 3. Rabbit's blood laked with distilled water (1 in 5).

Time (hrs.)...	0	24	48	144	216
Whole blood:					
Inorganic PO_4 (mg. P)	5	11·1	21·8	32·0	33·3
Sugar (mg.)	126	124	147	(99)?	143
Corpuscles:					
Inorganic PO_4 (mg. P)	(5·4)	45·4	86·7	89·3	—
Sugar (mg.)	53	44	47	68	—
Plasma:					
Inorganic PO_4 (mg. P)	5·4	5·4	5·8	—	—
Sugar (mg.)	128	128	128	—	—

In general it was found that no significant change in the reducing power occurred during the early stages of the autolysis in laked blood or corpuscles, although 50 % of the phosphoric esters might be hydrolysed. In the later stages of the autolysis a rise of about 30 % in the sugar value was usually observed. In one experiment with diluted plasma no change in the sugar value was found after 48 hours at 38°.

An increased sugar content could be explained by the hydrolysis of hexosephosphoric esters which possess a lower reducing power than that of the equivalent amount of free hexose, but the matter is not so simple as this. The reducing group is not liberated simultaneously with the phosphate and

may be derived from an entirely different compound by a reaction having no connection with the other. The presence in the blood of "combined sugar" (or "protein sugar") which can be set free by acid hydrolysis has been noted by many workers [cf. Condorelli, 1924]. For the present we retain an opinion that this sugar is combined on the one hand with phosphoric acid and on the other—through the potential aldehydic or ketonic group—with some other molecule, these bonds being hydrolysed by different enzymes at different rates. The evidence for this is admittedly slight and further investigation is desirable.

Possible changes of the sugar content were also looked for during the hydrolysis of blood filtrates by bone extracts. Since hexosemonophosphoric ester possesses 66 % of the reducing power of its equivalent in hexose, the liberation of inorganic phosphate equivalent to 6 mg. P from this compound would give an increased reducing power equal to 12 mg. glucose. Such increases were actually observed in one or two experiments, but in others no significant change was found and we concluded that under the conditions of the experiments the possible changes were too small to be satisfactorily demonstrated.

Changes in unlaked blood.

The laking of whole blood or corpuscles with distilled water produces two marked results, the rapid hydrolysis of the phosphoric esters and the inhibition of glycolysis, followed after 24–48 hours by an increase in the reducing sugar. In unlaked blood very little change occurs in the inorganic phosphate but the sugar rapidly disappears. In order to obtain more evidence as to the possible connection between these two reactions we investigated the behaviour of unlaked blood under slightly varying conditions of p_H.

Exp. 1 was carried out with rabbit's blood (mixed arterial and venous, containing oxalate); 2 cc. of whole blood, corpuscles or plasma were measured into tubes which were kept at 38° for 3 hours without antiseptic, the inorganic phosphate and sugar being thus estimated. The results are given in mg. per 100 cc.

Exp. 1.

	0 hours		3 hours	
	H_3PO_4 mg. P	Sugar mg.	H_3PO_4 mg. P	Sugar mg.
Whole blood	5·46	119	6·36	57
Do. shaken out with air	5·46	119	5·24	70
Do. + ·01 cc. NaOH	5·46	119	5·40	70
Corpuscles	5·36	52	16·7	21
Plasma	5·54	128	5·54	132

In this experiment an appreciable hydrolysis of organic phosphate occurred in the whole blood as taken from the animal but was entirely inhibited by the addition of a very small amount of alkali, or by shaking out with air, which would likewise raise the p_H. In the latter case a small decrease in free phosphate was noted. Glycolysis occurred in both whole blood and corpuscles but not in the plasma.

The effect of small additions of alkali and of acid was further tested in the following two experiments, in which changes in unlaked and laked blood were simultaneously investigated. Defibrinated rabbit's blood was used. Results are given in mg. per 100 cc. blood.

Exp. 2.

	Hours at 38°	Unlaked blood: Inorganic phosphate mg. P	Unlaked blood: Sugar mg.	Blood laked with distilled water (1 in 5): Inorganic phosphate mg. P	Blood laked with distilled water (1 in 5): Sugar mg.
0. Whole blood	0	4·79	116	—	—
1. Defibrinated blood (D)	0	4·54	115	—	—
2. 2 cc. D + ·045 cc. *N* H_2SO_4	2	7·10	98	8·6	(99)?
3. „ + ·030 cc. „	2	5·83	94	10·1	105
4. „ + ·015 cc. „	2	4·16	77	10·1	105
5. „ + ·005 cc. „	2	4·06	73	—	—
6. „ + ·005 cc. *N* NaOH	2	3·56	75	8·4	95
7. „ + ·015 cc. „	2	3·62	73	8·4	96
8. „ + ·030 cc. „	2	3·80	61	6·4	88
9. „ + ·045 cc. „	2	5·03	69	6·0	88

Exp. 3.

0. Whole blood	0	5·98	116	10·0	—
1. Defibrinated blood (D)	0	5·70	118	—	—
6. 2 cc. D + ·005 cc. *N* NaOH	2	4·94	72	—	—
6 *a*. „ + ·005 cc. „	3	5·47	68	—	—
8. „ + ·030 cc. „	2	5·23	74	—	—
8 *a*. „ + ·030 cc. „	3	5·70	79	—	—
10. „ + ·04 cc. „	2	—	—	9·43	103
11. „ + ·07 cc. „	2	—	—	6·71	104
12. „ + ·10 cc. „	2	—	—	6·71	104
13. Laked blood without acid or alkali	2	—	—	10·06	107

In both experiments there was a definite reduction in the amount of inorganic phosphate on defibrination, which was also noted by Lawaczeck. A further marked reduction occurs when the blood is kept during 2 hours at 38°, the amount of this reduction being greatest in the presence of small amounts of added alkali. (The defibrinated blood, which has been shaken with air, with a low CO_2 content, is itself more alkaline than normal blood.) This decrease in free phosphate must indicate a synthesis of organic phosphorus compounds, taking place in unlaked whole blood most readily when the p_H is above the normal. Exp. 3 shows that this synthetic power is only retained for a limited period after withdrawal of the blood from the body, the decrease in free phosphate being less after 3 hours than after 2 hours. This also is in agreement with Lawaczeck's findings.

When the p_H rises above or falls below certain limits, the rate of hydrolysis of the phosphoric esters becomes greater than the rate of synthesis, and the inorganic phosphate increases. At a p_H somewhat lower than 7·0 the rate of hydrolysis may approach that in laked blood.

Lawaczeck's interesting findings on the effect of passing carbon dioxide or pure air through the blood would seem to be due to the alteration of p_H thus brought about.

We have obtained similar results with blood shaken up several times with carbon dioxide or with air free from carbon dioxide and then kept in a corked flask at 38° for 2 hours.

		Inorganic phosphate mg. P	Sugar mg.
0.	Whole blood	5·65	112
1.	Defibrinated blood	5·41	112
2.	„ „ shaken with pure air	5·44	63
3.	„ „ „ „ CO_2	12·82	106
4.	„ „ „ „ CO_2 and kept for 1 hour at 38° ...	9·05	107
5.	„ „ „ „ CO_2 „ „ 38° and then shaken with pure air and kept for a further 1 hour at 38° ...	6·25	95

The variations in the amount of reducing sugar under the above conditions are also very interesting. When the synthesis of phosphoric esters is greatest considerable glycolysis occurs, although the converse is not necessarily true. On the other hand, when rapid hydrolysis is taking place the glycolysis is much less rapid or may be entirely absent. One cannot, however, draw the conclusion that the two reactions—esterification of phosphoric acid and synthesis of non-reducing compounds from sugar—are necessarily connected, since two independent enzyme reactions, both affected similarly by the p_H, might well show parallel variations of this kind. Recent work by Kay [1924] has shown that during acidosis produced in a human subject by the ingestion of ammonium chloride the amount of phosphoric esters in the venous blood was considerably diminished. It seems possible that *in vivo* as *in vitro* a delicate equilibrium exists between the synthetic and the hydrolytic activities of the blood enzyme and that this is markedly affected by very slight changes in the reaction of the blood.

The whole of the evidence of our experiments is not sufficient to prove that the phosphoric esters and esterase of the blood actually play a part in carbohydrate metabolism, but we think that the experimental facts are very suggestive and are worth further investigation and confirmation.

REFERENCES.

Condorelli (1924). *Il Policlinico*, **31**, 125.
Hagedorn and Jensen (1923). *Biochem. Z.* **135**, 46.
Jones and Perkins (1923). *J. Biol. Chem.* **55**, 567.
Kay (1924). *Biochem. J.* **18**, 1133.
Kay and Robison (1924, 1). *Biochem. J.* **18**, 755.
—— (1924, 2). *Biochem. J.* **18**, 1139.
Lawaczeck (1924). *Biochem. Z.* **145**, 351.
Martland and Robison (1924). *Biochem. J.* **18**, 765.
Robison and Soames (1924). *Biochem. J.* **18**, 740.

CLI. THE POSSIBLE SIGNIFICANCE OF HEXOSE-PHOSPHORIC ESTERS IN OSSIFICATION. PART IV. THE PHOSPHORIC ESTERS OF THE BLOOD.

PRELIMINARY COMMUNICATION.

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The suggestion that hexosephosphoric esters in conjunction with the bone enzyme are concerned in the deposition of calcium salts in bone [Robison, 1923] led us to attempt the isolation and identification of the organic acid-soluble phosphorus compounds in blood in order to test the correctness of the surmise. This work has been carried on during the past 18 months, and, as it seems probable that some months of further work will be required before we can publish our results in full, we wish to give this very brief preliminary account.

The first problem was to find the best protein precipitant which would not interfere in the further steps in the isolation of the esters. After many trials trichloroacetic acid was finally adopted and has proved very satisfactory, although somewhat costly when used in large amounts.

The anticoagulant—fluoride or oxalate—used in taking the blood was a source of much trouble until we adopted the method recommended in another paper [Martland and Robison, 1924] of taking the blood directly into 1 % trichloroacetic acid and then adding more of the acid to precipitate the proteins. The neutralised protein-free filtrate was treated with basic lead acetate and the lead salts decomposed with H_2S. Very early in our work we obtained in this way solutions which contained organic phosphorus, reduced copper salts, and were laevorotatory. We were, however, not satisfied that these properties were due to the same substance.

From these solutions two barium salts of phosphoric esters were obtained.

A. Practically insoluble in water and very sparingly soluble in cold dilute acids, soluble in warm hydrochloric acid. Did not reduce Fehling's solution. This salt formed the greater part of the total organic phosphate and contained those esters which were not hydrolysed by the bone enzyme [Kay and Robison, 1924].

B. Readily soluble in water, reduced Fehling's solution, and was laevorotatory. The ratio $\frac{\text{Reducing power as glucose}}{\text{P}}$ for this salt was very similar to that found for hexosemonophosphoric ester [Robison, 1922].

The phosphorus content of the salt was, however, only about one-third of that required for barium hexosemonophosphate. Fractional precipitation by alcohol, precipitation by mercuric acetate and finally extraction of the acid solution with butyl alcohol failed to alter greatly the ratios of reducing power (MacLean's method) to P and to the rotatory power, nor did the P content of the salt rise. Either the ester is mixed with some other compound whose salts possess very similar solubility, or else other groups of considerable molecular weight are attached to the hexosephosphate we believe to be present.

The ratios of the purified ester are

$$\frac{\text{Reducing power as glucose}}{\text{P}} = 4{\cdot}6.$$

$$\frac{\text{Rotation in 400 mm. tube (Hg green line)}}{\text{Reducing power as glucose}} = -26.$$

$$\frac{\text{Rotation in 400 mm. tube (Hg green line)}}{\text{P}} = -118.$$

Finally, 90 % of this ester was hydrolysed in six hours at 38° (p_{H} 8·4) by a 5 % extract of rat bones. The conditions were those adopted in the investigation of blood filtrates [Kay and Robison, 1924] and it is thus proved that this reducing substance is (or contains) the phosphoric ester forming the fraction hydrolysed by the bone enzyme and probably concerned in the ossification process.

Until the question of homogeneity has been settled it is, of course, impossible to say definitely that the phosphoric ester is identical with the reducing compound, but this is most probably the case. It is perhaps less improbable that the laevorotation should be due to a second unknown compound mixed with the ester. The purification and examination of these two compounds is being carried on, and it is hoped to publish a fuller account of them in a few months.

REFERENCES.

Kay and Robison (1924). *Biochem. J.* **18**, 755.
Martland and Robison (1924). *Biochem. J.* **18**, 765.
Robison (1922). *Biochem. J.* **16**, 809.
—— (1923). *Biochem. J.* **17**, 286.

CLII. THE CHANGES IN THE LIME CONTENT OF THE HEN'S EGG DURING DEVELOPMENT.

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Prout [1822], who was unable to detect lime in the contents of the egg before development, but observed its presence in the newly-hatched chick, concluded that the lime was derived from the shell. Though Voit [1877] could not repeat this result, it was confirmed by Vaughan and Bills [1878]. They found that the fresh egg contained an average of 0·07 g. $CaSO_4$ (= 0·03 g. CaO) and the hatched chick 0·38 g. $CaSO_4$ (= 0·16 g. CaO). The opposite was again observed by Pott and Preyer [1882]. Tangl [1908], from analyses of the shells of fresh eggs and of those from hatched chicks, concluded that both organic matter (0·4 g.) and lime (0·16 g.) disappeared from the shell. The most satisfactory data upon the lime content of the egg and of the developing chick are those of Carpiaux [1908]. Carpiaux determined the lime content of the egg at several stages of development and found that it rose from 0·04 g. to 0·20 g., the first noticeable increase occurring about the thirteenth day. His analyses of the shell both before and after development did not show any particular change in its composition. Although the increase of lime in the embryo had thus been clearly demonstrated, it was considered that some further information might still be gained. Analyses of the lime content at each day of incubation have now been made, both as regards the interior including the chick and yolk sac, the shell and the membrane. Our results confirm those of Vaughan and Bills and of Carpiaux, that lime enters the egg from the shell. The loss of lime from the shell could not be detected with certainty owing to the great variations of its lime content, but the membrane, at first almost lime-free, becomes richer in lime. An increase in lime has also been found in pigeons' eggs during development, so that it is very probable that lime passes from the shell to the contents in all birds' eggs during development. It has further been found that the membrane of soft shelled eggs contains large amounts of lime, more than sufficient for the needs of the developing chick. In all cases the shell has much more lime than is needed. Attempts to hatch soft-shelled eggs were a failure owing to the contents drying up. The function of the shell is thus partly protective against breakage, to furnish lime and to prevent evaporation.

Experimental.

The experiments were designed so as to determine the total lime in the contents of eggs at each day of incubation. At the later stages, at which separation of embryo and yolk sac becomes possible, the lime was to be estimated in each of these parts. With any increase of lime in the interior, a corresponding loss would be expected from the shell. This loss would be expected to involve loss of carbon dioxide, as the shell is composed of 90–95 % calcium carbonate with traces of magnesium carbonate, phosphates and organic matter. The shell was therefore also examined for lime and carbonate. Owing to the great variations which were found in the amount of lime, carbon dioxide and weight of membrane, these estimations were abandoned as it was not possible to tell if lime and carbon dioxide were actually lost by the shell. It may be pointed out that Tangl, though he used eggs from the same hen, also found variations both in weight of shell and membrane, and really arrived at his conclusion of loss of lime from the shell by calculation. In the later experiments the egg membrane was analysed since, if lime enter the egg from the shell, it must pass through the membrane.

Two dozen eggs were required for the series of analyses; two were usually analysed before incubation, the others at daily intervals, with the extra egg for a possible longer period of development with an incubator temperature lower than the normal of the hen. Three series of eggs had to be used. The first series, O, were eggs from the experimental group of Light Sussex chickens of Plimmer and Rosedale [1923], fed upon a diet of white rice, dried yeast, slaked lime and cod liver oil. These eggs were all unfertile and were thus of no value for this investigation. The second series, S, also Light Sussex, were from a dealer from hens fed upon another diet. Many of these were unfertile, and most of the embryos were dead on removal of the egg from the incubator. The third series, R, were from White Leghorn hens fed upon maize meal, middlings, bran, fish meal and dried yeast with an allowance of about 3 oz. per day of wheat. In this series the eggs with dead embryos could be discarded and replaced by other eggs which were then incubated for the necessary length of time. A complete series was thus obtained.

Preparation of the material. The eggs were weighed before incubation and on the day of removal from the incubator. The contents were separated from the membrane and shell, and the shell washed free from adhering contents with distilled water. The contents and water were put into a 700 cc. Jena glass flask, in which any small fragments of shell could be seen and removed, if necessary. In those cases in which embryo and yolk sac were separately analysed, they were cut apart with scissors and the wash water added to the yolk sac portion. The shell was allowed to dry in the air and weighed. In the earlier experiments the whole was used for carbon dioxide and lime estimations and the membrane afterwards collected, dried and weighed, so as to get the weight of shell. In the later experiments the shell was picked from the

membrane; this was a simpler operation with the eggs of the later periods of incubation; the shell then easily chips from the membrane which at the same time becomes more opaque and tougher in texture.

Estimation of lime. The lime in the contents of the eggs was estimated by Aron's process as adapted by Plimmer and Page [1913] as calcium sulphate, by precipitation, after oxidation with sulphuric and nitric acids, with two volumes of alcohol. The same method was used for the estimation of the lime in the egg shell and membrane, the former after the estimation of the carbon dioxide.

Estimation of carbon dioxide in the shell. The method adopted for this purpose was that of Van Slyke [1918], but it required some modification. The method consists in liberating the carbon dioxide by dilute hydrochloric acid in an evacuated flask and absorbing the gas in baryta water, which is afterwards titrated. In the first place it was necessary to use the whole shell on account of variations in its thickness and for the recovery of the whole membrane as well as for the subsequent estimation of the lime. The dried shell was placed in a tall beaker of 550 cc. capacity and covered with an inverted funnel without stem. A second and larger funnel with short stem was placed on the top of the beaker with the stem passing into the inverted funnel below. The beaker was put into a large crystallising dish containing 100 cc. of N sodium hydroxide. The whole was then put into a large vacuum desiccator with an opening at the top. This was fitted with a rubber stopper holding a tap funnel and a tube with rubber tubing and screw clip. The desiccator was then exhausted and closed by tightening the screw clip. Very dilute hydrochloric acid was now allowed to drop in from the tap funnel at first slowly on account of the rapid reaction and frothing. After this had ceased stronger acid was allowed to run in until the whole of the carbonate was decomposed. The contents were usually allowed to stand for 24 hours to complete the absorption of the carbon dioxide by the sodium hydroxide. Air was then admitted, the contents of the crystallising dish titrated with $N/2$ hydrochloric acid, using the B.D.H. Universal Indicator instead of methyl orange and phenolphthalein. The first reading was taken when all the blue colour was discharged and a sharp green appeared without trace of yellow. At this point the p_H is 8–8·5, the same as the neutral point of phenolphthalein, and corresponds to conversion of carbonate into bicarbonate. The second reading was taken when all orange colour was discharged and the solution became red. The reaction corresponds here to p_H 3, the neutral point of methyl orange. Both end points were quite sharp and distinct. The difference between the reading corresponds to half the carbon dioxide in terms of $N/2$, from which the carbon dioxide in the whole shell was easily calculated.

The solution containing the lime as calcium chloride was separated from the undissolved membrane by simply picking this out and washing it, filtered from particles of pigmented matter and made up to 1000 cc. The membrane was dried and weighed. The lime was estimated in 100 cc. by adding 20 cc.

Egg Series O.

The first four were from one hen, the next two from another, the last two from a third.

Contents			Shell				
Weight g.	Lime g.	Lime %	Weight g.	Membrane g.	CO_2 g.	Lime g.	Lime in shell-less membrane %
44·6	0·0367	0·08	6·4	0·2	2·18	2·76	44·8
43·4	0·0374	0·09	5·6	0·2	2·28	2·71	49·9
45·1	0·0377	0·08	5·9	0·1	2·37	3·03	52·6
45·9	0·0370	0·08	6·1	0·2	—	3·1	52·3
49·4	0·0412	0·08	5·6	0·3	2·04	2·6	49·1
50·0	0·0360	0·07	5·0	0·2	1·84	2·51	52·2
50·6	0·0364	0·07	5·4	0·1	—	2·75	52·2
50·4	0·0400	0·08	5·6	0·2	2·09	2·81	52·0

Egg Series S.

Days of incubation	Contents			Shell				
	Weight g.	Lime g.	Lime %	Weight g.	Membrane g.	CO_2 g.	Lime g.	Lime in shell-less membrane %
0	57·4	0·0463	0·08	5·4	0·4	1·90	2·65	53·4
0	49·8	0·0328	0·07	6·0	0·1	2·30	2·82	48·1
9 unfert.	50·9	0·0450	0·09	5·3	0·3	2·09	2·65	52·8
13 „	56·6	0·0443	0·08	6·3	0·2	2·37	3·06	49·8
19 „	55·4	0·0534	0·09	4·7	—	—	—	—
0	50·5	—	—	—	0·4	2·05	2·25	47·8
7? fert.	49·2	0·0419	0·09	5·7	0·3	2·28	2·73	50·8
8? „	55·0	0·0462	0·08	6·1	0·3	2·41	3·04	52·7
14? „	60·6	0·0400	0·07	5·6	0·3	2·11	2·77	52·8
15? „	55·8	0·0514	0·09	6·2	0·3	2·29	2·97	50·0
20 live	49·4	0·1427	0·29	5·6	0·1	2·42	2·88	51·6
? dead	57·0	0·1282	0·22	6·0		Lime g.		Lime g.
? „	57·8	0·1381	0·24	6·0	Chick	0·0726	Yolk sac	0·0655
? „	52·4	0·1756	0·35	5·9		0·1016		0·0740
? „	53·8	0·1448	0·27	4·9		0·0622		0·0826
? „	—	0·0649	—	—		0·0170		0·0479

Five other unfertile eggs had an average lime content of 0·0397 g.

Egg Series R.

Days of incubation	Weight of contents	Lime in contents				Weight of shell	Weight of membrane	Lime in membrane
		Chick	Yolk sac	Total				
	g.	g.	g.	g.	%	g.	g.	g.
0	52·3	—	—	0·0536	0·10	5·1	0·2	0·0002
1	53·4	—	—	0·0508	0·09	5·0	0·3	—
5	59·8	—	—	0·0509	0·09	4·2	0·2	—
6	66·0	—	—	0·0400	0·06	4·5	0·2	0·0008
7	63·9	—	—	0·0487	0·08	3·9	0·3	—
8	62·5	—	—	0·0394	0·06	6·0	0·5	0·0025
9	63·0	—	—	0·0514	0·08	4·0	0·2	0·0008
10	56·4	trace	0·0492	0·0492	0·09	5·6	0·2	0·0020
11	58·8	0·0021	0·0544	0·0565	0·10	5·4	0·1	0·0025
	55·0	—	—	0·0579	0·10	5·5	0·2	0·0054
12	48·4	0·0015	0·0385	0·0400	0·08	4·2	0·1	0·0008
	49·5	—	—	0·0443	0·09	6·3	0·3	0·0019
13	51·2	0·0035	0·0566	0·0601	0·11	5·3	0·3	0·0029
14	52·4	0·0063	0·0422	0·0485	0·09	5·8	0·2	0·0050
	53·9	—	0·0509	0·0509	0·09	4·6	—	—
15	54·9	0·0192	0·0590	0·0782	0·14	6·4	0·1	0·0038
16	56·6	0·0331	0·0600	0·0931	0·16	6·4	0·2	0·0080
17	48·8	—	0·0700	—	—	5·7	0·2	0·0086
18	51·1	0·0782	0·0522	0·1304	0·25	5·0	0·2	0·0062
19	57·7	0·0988	0·0727	0·1715	0·30	6·0	0·2	0·0073
20	52·0	0·0629	0·0531	0·1160	0·22	5·2	0·2	0·0082
21	54·5	—	—	0·1816	0·33	4·5	0·2	0·0058
	58·7	0·1262	0·0646	0·1908	0·33	5·6	0·3	0·0073

Light Sussex hatched chick, 0·2407 g.

Pigeons' eggs.

	Weight of contents	Lime			Shell weight	Membrane	
		Chick	Yolk sac	Total		Weight	Lime
	g.	g.	g.	g.	g.	g.	g.
Unfertile	16·3	—	—	0·0111	1·2	0·1	0·0003
,,	17·3	—	—	0·0107	1·2	0·1	0·0004
,,	12·2	—	—	0·0074	1·3	0·1	trace
,,	12·8	—	—	0·0103	1·0	0·1	,,
,,	13·3	—	—	0·0080	1·2	0·1	,,
,,	14·7	—	—	0·0109	1·3	0·1	,,
,,	15·9	—	—	0·0092	1·3	0·1	0·0004
,,	20·1	—	—	0·0174	1·4	0·2	trace
Fertile	17·9	—	—	0·0091	1·6	0·1	0·0005
,,	17·8	—	—	0·0100	1·4	0·1	0·0005
,,	15·2	0·0114	0·0132	0·0246	1·3	0·1	0·0008
,,	16·2	0·0077	0·0128	0·0205	1·5	0·1	0·0011

Distribution of lime in egg contents and membrane.

Contents					Membrane		
Yolk	White	Total	Yolk	White	Weight	Lime	Lime
g.	g.	g.	%	%	g.	g.	%
0·0418	0·0010	0·0428	97·7	2·3	0·12	0·0002	0·2
0·0375	0·0011	0·0386	97·1	2·9	0·11	0·0006	0·5
0·0304	0·0035	0·0339	89·7	10·3	0·15	0·0006	0·4
0·0310	0·0056	0·0366	84·7	15·3	0·18	0·0005	0·3
0·0384	0·0026	0·0410	93·7	6·3	0·18	0·0010	0·6
0·0281	0·0008	0·0289	98·9	1·1	0·16	0·0001	0·1

Lime in double eggs and shell-less eggs.

Double	Contents	Lime	Shell-less	Contents	Lime	Membrane	Lime	
g.	g.	g.	g.	g.	g.	g.	g.	
77·5	—	0·0788	—	—	—	0·98	0·3142	
74·3	—	0·0793	—	48·1	0·05	1·03	0·399	
			—	—	—	4·0	0·581	
			—	3·0	0·0317	0·67	0·1604	
			—	—	—	3·25	0·75	(from goose)

of sulphuric acid and 200 cc. of alcohol, collecting the precipitated calcium sulphate, drying and weighing.

The figures of analysis are given in the tables. Those of the S series of eggs are curtailed and many referring to unfertile eggs of the R series are not included.

Though some eggs have been found to have a slightly higher lime content, the mean value for unincubated and unfertile eggs from the large number here examined may be taken as 0·04 g. A distinct increase in this amount was first shown at the 11th day of incubation (0·06 g.); the increase became quite definite at the 15th day (0·08 g.). Subsequently there was a daily increase amounting to 0·02 g. A chick about to hatch had 0·2 g. and a hatched chick 0·25 g. The final total is five to six times the original content. The gradual increase is clearly observed in the content of the chick, the amount in the yolk-sac remaining nearly constant, being scarcely double that of the original egg. As the yolk sac is in contact with the shell during development, this structure must play the active part in the transference of the lime; as

soon as lime passes from it into the chick, it must take up fresh lime from the shell. The mechanism of the absorption of lime from the shell is difficult to prove. It is most simple to think that the carbon dioxide and water which are given off during incubation produce calcium bicarbonate. Egg membranes are excellent dialysers and allow calcium bicarbonate to pass through. The passage of the bicarbonate would thus be in the opposite direction to that of the escaping carbon dioxide. In the work of Plimmer and Scott [1909] on the changes in the phosphorus compounds in the developing egg, inorganic phosphoric acid was detected at the 13th day and was present in increasing amounts during the rest of the development. The two processes seem to run parallel. It would therefore seem more likely that the phosphoric acid circulating in the yolk sac, which is in contact with the shell membrane, becomes neutralised by solution of lime from the shell. The passage of lime through the membrane is definitely shown by the increase in its lime content. There are traces only at the beginning whilst during the last seven days the amount is considerable. The presence of lime salts in the membrane is probably the cause of its opaque appearance during the last week of incubation.

The corresponding loss of 0·2 g. from a total of 2·5–3·0 g. in the shell, an amount of lime corresponding to 1/10 of the total, was not discovered. The analyses of the shell were discontinued after the great variations were found in the series O and S in which so many eggs were unfertile, or the chicks dead. If they had been continued, the loss from the shell would most probably have been found.

The results of the increase in lime in the egg are confirmed by the analyses of pigeons' eggs; no data were available as to their exact age, the eggs being deserted by the parents. The increase was up to twice the original figure.

Shell-less eggs have been unexpectedly found to contain large amounts of lime, much more than is required for the growth of the embryo. It was found impossible to incubate these eggs, as they invariably dried up in the course of a week. The shell has thus probably the function of preventing evaporation of water from the egg, in addition to supplying lime and generally acting as a protective covering. The large excess of lime in the shell of eggs points to non-hatchability as being due to some cause within the egg.

It may finally be noted that double eggs contain twice the normal amount of lime and the lime is present mainly in the yolk; the lime content of the yolk varied from 85 to 99 % of the total.

Summary.

The lime content of eggs has a mean value of about 0·04 g.

The lime content of eggs increases to 0·20–0·25 g. during development of the chick. The first increase is noticeable at the 11th day of incubation and the subsequent increase amounts to about 0·02 g. per day until the chick is hatched.

A similar increase occurs in pigeons' eggs during development.

The lime and carbon dioxide content of the egg shell were found to be very variable.

The membrane of the shell also shows an increase of lime during incubation, which shows that lime passes through the membrane.

Shell-less eggs contain more than sufficient lime for the needs of the developing chick. The shell will thus act in preventing loss of water as well as in supplying lime for the chick.

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REFERENCES.

Carpiaux (1908). *Bull. Acad. Roy. Belg.* 283.
Plimmer and Page (1913). *Biochem. J.* **7**, 157.
Plimmer and Rosedale (1923). *Biochem. J.* **17**, 790.
Plimmer and Scott (1909). *J. Physiol.* **38**, 247.
Pott and Preyer (1882). *Pflüger's Arch.* **27**, 353.
Prout (1822). *Phil. Trans. Roy. Soc. Lond.* 377.
Tangl (1908). *Pflüger's Arch.* **121**, 423.
Van Slyke (1918). *J. Biol. Chem.* **36**, 351.
Vaughan and Bills (1878). *J. Physiol.* **1**, 434.
Voit (1877). *Zeit. Biol.* **13**, 518.

CLIII. THE RELATIONSHIP OF PHOSPHATES TO CARBOHYDRATE METABOLISM.

I. TIME RELATIONSHIP OF THE CHANGES IN PHOSPHATE EXCRETION CAUSED BY INSULIN AND SUGAR.

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Accompanying changes in the metabolism of sugar, variations in the inorganic phosphate content of the blood, and in the excretion of phosphate in the urine, have been observed by several investigators. Fiske [1920] found that ingestion of sugar resulted in a temporary diminution in the urinary output of phosphorus followed by a compensatory increase, and more recently Blatherwick, Bell and Hill [1923] have reported that the inorganic phosphates of the blood and urine become diminished after giving glucose for tolerance tests. It has also been shown that the administration of insulin is followed by a decrease in the inorganic phosphate of the blood. This was first observed in rabbits by Wigglesworth, Woodrow, Smith and Winter [1923], Winter and Smith [1924]. The same change in the blood of normal and diabetic individuals, accompanied by a decrease in excretion of phosphate in the urine, has been found by Blatherwick, Bell and Hill [1923], Perlyzweig, Lathan and Keefer [1923] and Harrop and Benedict [1923, 1924]. Another significant fact has been reported by Elias and Weiss [1922], to the effect that the intravenous injection of phosphate solutions is followed by a reduction of the blood sugar, in cases in which this is above the normal level as in diabetes or in alimentary hyperglycaemia.

The present paper presents further evidence to show the existence of a close relationship between phosphorus and the metabolism of carbohydrate. It is the outcome of an investigation which was started to test the possibility of the occurrence of a disturbance of the acid base equilibrium during insulin hypoglycaemia. Such striking changes in phosphate excretion were met with, that attention was directed to this feature. In order to determine the nature of the metabolic changes which might be responsible, analysis of the urine was also made for total nitrogen, ammonia and creatinine.

Methods.

Observations were made on normal and depancreatised animals, which were starved throughout the experiment, so that the effect of food might be eliminated. The animals were catheterised at intervals of 3 hours during the day, to obtain the urine in fractional quantities and the night urine was collected as a whole, in metabolism cages. 50 cc. of water were given by stomach tube at the beginning of each of the day periods, and 150 cc. were given at night. The bladder was irrigated with 30 cc. water at the end of each catheterisation, and no evidence of infection or bladder irritation developed during the experiments. Chloroform was added to the samples of urine and these were kept at low temperature. The analytical methods used were: for phosphate, that of Briggs; for sugar, that of Shaffer and Hartmann; for nitrogen, ammonia and creatinine, those of Folin; and for titratable acidity, that of Fiske. The results are expressed graphically, those of one experiment being also given in tabular form. In the graphs the vertical lines mark equal periods of 3 hours, beginning with 10 a.m. on each day. The horizontal lines represent the amounts of the various substances excreted per hour during each of the periods. The figures on the curves give the total amount of the corresponding substance excreted for each day. The total excretion is calculated to the 24-hour value when the period is not exactly of this duration.

Results.

I. *Normal animals.*

Dog. B. Female, weighing 12·0 kilos. at the commencement of the experiment, was observed from March 4th until March 12th, following a preliminary

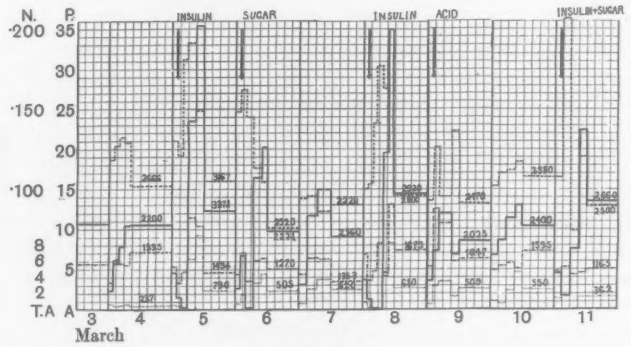

Fig. 1. Normal dog *B.* Phosphorus ———. Nitrogen – – – –. Ammonia –·–·–·–. Titratable acidity .....

period of 3 days' fasting. The total nitrogen, ammonia, acid and phosphorus were determined at the usual periods, insulin or sugar being given on certain days.

On the normal days, the excretion of nitrogen per hour was somewhat

higher during the day than during the night, the highest values being usually reached in the early afternoon. The total daily excretion did not change significantly during the period of observation. The phosphorus excretion was at its lowest in the forenoon hours, increased during the remainder of the day, and reached its highest in the evening, the output for the night being considerably higher than that for the day. This diurnal variation has been observed in man by Fiske [1921], Campbell and Webster [1921] having also noted a higher excretion of phosphate during the night than during the day. The ammonia and the acidity varied in the same direction as the phosphorus.

Insulin was given at 12 noon on the second day, March 5th, the dose being insufficient to cause severe symptoms of hypoglycaemia although the dog became lethargic and inactive for a few hours after the injection. The excretion of phosphorus fell from 4·54 to 1·42 mg. per hour in the 1st period of $2\frac{1}{4}$ hours after the injection and to 0·18 mg. per hour in the 2nd period of $1\frac{3}{4}$ hours. This was accompanied by a corresponding reduction in the acidity and ammonia, but by no immediate change in total nitrogen. During the 2nd period of $1\frac{3}{4}$ hours the nitrogen rose markedly, accompanied by a lesser rise in ammonia, the acidity, however, continuing to fall with the phosphate. In the 3rd period, between the 4th and 7th hours following the injection of insulin, a most remarkable increase occurred in the excretion of phosphorus, and the acidity and ammonia rose in sympathy. In the next period (7th to 10th hours following insulin) the phosphorus rose still higher but the total nitrogen began to decline, with the ammonia following the latter, and the acidity the former. In the night urine the phosphorus remained above the normal, so that in the total 24-hours' urine it amounted to 337 mg., which, when compared with that observed on the normal days for this animal (4th, 7th and 10th), is approximately 45 % above the average. The total nitrogen fell to about its normal level during the night, but was increased for the 24 hours. The ammonia and the acidity for the 24 hours were decidedly greater than the normal.

Insulin was again given to this animal on March 8th with the same general results. The decrease in phosphorus was, however, more marked, and traces only could be detected in the samples of urine collected in the first 6 hours after insulin, the post-hypoglycaemia peak being reached in about 10 hours. The nitrogen excretion rose in the 2nd period after insulin, the peak being reached in the 3rd period, i.e. about 8 hours after the injection. The ammonia fell in the 1st period, when the phosphate was low, then rose in the 2nd, when the nitrogen was rising, reaching its peak, later, when the phosphate was at its highest. The acidity ran parallel with the phosphate throughout. The total quantities of phosphorus, nitrogen and ammonia for the 24 hours were greater than normal. The volume of urine was found to be diminished during the hypoglycaemic period on both insulin days, this being followed by a compensatory increase occurring in from 7 to 12 hours after the injection. On March 15th the urine was reduced from 9 cc. per hour before insulin to

5 cc. per hour in the first 4 hours following it, and it rose to 20 in the next 3 hours. On March 8th the excretion was reduced from 13 to 8 and 5 cc. per hour in the first 4 hours, rising to 32 between the 6th and 8th hours.

On March 6th the dog was given 50 g. of glucose (approximately 5 g. per kilo.) by stomach tube. The phosphate excretion which continued its usual afternoon increase in the 1st period ($2\frac{1}{4}$ hours) fell to 0·14 mg. per hour in the 2nd period. Then a large increase occurred, lasting about 5 hours. The total 24-hour output was not greater than the average normal output. The nitrogen excretion gradually diminished, reaching its lowest level during the night, and on the following day it was considerably below the average. The ammonia and titratable acidity followed the changes of the phosphate.

On March 11th, at 12.55 p.m., 50 g. glucose were given along with 30 units of insulin. Owing to vomiting it is not known how much sugar was absorbed. The phosphate excretion fell to 1·84 mg. then rose to 22·30 mg. per hour, the total for the day being somewhat increased. The nitrogen showed a temporary increase but doubt exists as to whether this may not have been due to some vomitus having become mixed with the urine; later, a decrease occurred so that the total for the day was not changed. The ammonia was little changed and the acidity varied with the phosphate.

On March 9th, 100 cc. of $N/10$ H_2SO_4 were given by stomach tube. The phosphate excretion did not deviate from the normal and there was no increase in excretion for the day. The ammonia increased immediately, but the total for the day was very little more than the normal. The titratable acidity of the urine followed the usual course during the day, and was unaltered for the 24 hours.

Dog A. A small terrier was starved from March 6th to March 25th, during which period its weight declined from 7·0 kilos. to 5·5 kilos. Phosphorus and ammonia were determined in the usual fractions of the urine, but the nitrogen, only for the whole 24 hours, except for one day, March 20th.

On March 10th and 11th, which were control days, the excretion of phosphate per hour was a little lower in the morning than the average for the preceding night, but rose decidedly during the afternoon and evening. The same diurnal variation occurred on March 14th and March 21st, the days following insulin. The ammonia curve paralleled that of phosphate.

Insulin (10 units) was given at the beginning of the 1st morning period on March 13th. This resulted in almost complete disappearance of phosphate from the urine collected during the succeeding 6 hours; in the next 6 hours the excretion reached a very high level. The total for the day though 40 % above that of the following day (14th) was no greater than that of the first two control days.

A smaller dose of insulin (7 units), given on March 15th, and again on March 17th, was followed by the same changes in phosphates, except that the secondary increase was earlier in appearing. The rise had begun in the 2nd period, 3 to 6 hours after insulin, reaching its maximum in the 3rd period, 6 to 9 hours. It should be noted that on March 15th, although the morning value was quite low before the insulin was given, it was reduced still further

after the injection. A smaller dose (5 units), given on March 23rd, caused the same definite changes. On each of these three insulin days there was a small increase in the phosphate output for the 24 hours, but that of the following day was also high especially on the 16th and 24th.

The nitrogen output was decidedly increased on the days when insulin was given. Thus, on March 13th, 3·587 g. was excreted, as compared with 2·108 g. on the following day and 2·278 g. and 2·046 g. on the first two normal days. On March 15th the nitrogen was 2·829 g. On March 17th the increase in nitrogen was less evident, the total for the day amounting to only 2·335 g. compared with 2·008 g. and 1·980 g. on the preceding and following days, respectively. On March 23rd, when the small dose of insulin was given, the output was only slightly increased, amounting to 2·443 g. with 2·311 g. on the day following.

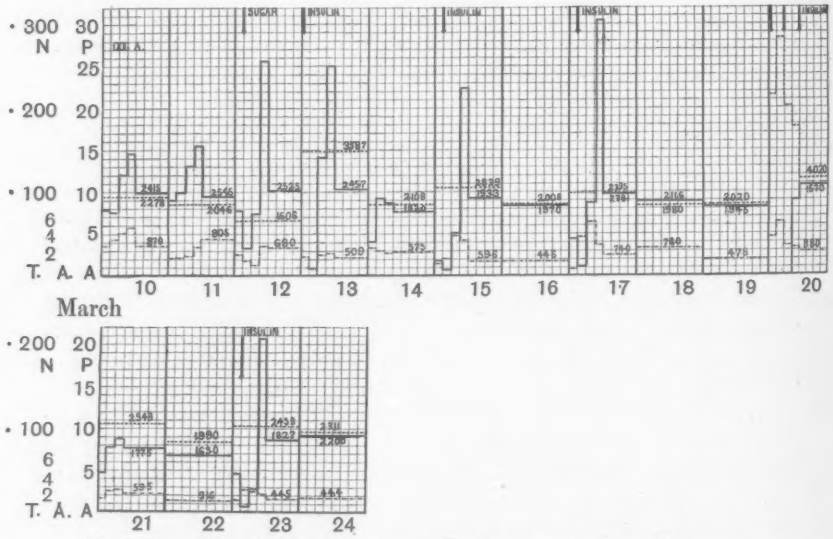

Fig. 2. Normal dog *A*. Phosphorus ———. Nitrogen – – – –. Ammonia –·–·–·–.

On March 20th, insulin was given three times (at 10 a.m., 8 units; at 4 p.m., 5 units; at 10 p.m., 3 units) without producing any symptoms, except lethargy. The first three fractions of urine collected during the day, did not contain phosphorus in measurable quantities. In the period from 7 to 10 p.m. the excretion had risen to 8·94 mg. per hour and there can be little doubt that a marked increase in phosphate occurred during the night. It is interesting to note, however, that the output of phosphorus for the 24 hours (156·93 mg.) was slightly below the normal (about 180 mg.); neither was there an increase on the following day. The nitrogen rose promptly to a very high level and during the 24 hours 4·064 g. were excreted. This represents an increase of

nearly 100 % over the normal output of 2·0 to 2·5 g. per day. The ammonia followed the total nitrogen.

On March 12th, 35 g. of glucose were given at 12.50 p.m. The phosphate excretion fell from 7·86 mg. per hour to 3·22 mg. in the next 3 hours, returning to 7·34 mg. in the following period. In the 3rd period, 6 to 9 hours after the ingestion of the sugar, the phosphate had risen to 26·60 mg., but the total for the day was not increased. The nitrogen output was markedly reduced, the decrease amounting to more than 20 %.

In two out of the four days in which a single dose of insulin was given a marked diminution was observed to occur in the volume of urine excreted. On March 15th and 23rd, the volume per hour fell to 4 and 2 cc. respectively during the hypoglycaemic period. Compensatory increases occurred several hours later. On one other day, March 19th, there was a smaller decrease, to 8 cc. from the normal rate of 12 or 13 cc.

This dog, on two or three occasions, passed a dark green viscous material by the bowel during the hypoglycaemic period. This was probably bile and intestinal secretion evacuated on account of the increased activity of the intestine, which is known to occur after insulin.

Dog F. A small fat terrier weighing 7·3 kilos. was subjected to a somewhat longer fast, from March 18th to April 10th. It stood the fasting well, though its weight fell to 4·0 kilos.

Fig. 3. Normal dog *F*. Phosphorus ———. Nitrogen – – – –. Ammonia –·–·–·–.

The excretion of nitrogen and phosphorus was fairly constant from day to day though a gradual reduction became evident towards the end. The usual

Dog F.

No.	Period	Time	Duration	Urine cc.	Total N. g. Total	Total N. g. Per hour	Ammonia N. mg. Total	Ammonia N. mg. Per hour	Ammonia N. mg. Per cent.	Creatinine mg. Total	Creatinine mg. Per hour	Phosphorus mg. Total	Phosphorus mg. Per hour
1	Mar. 20,	9.50 a.m.	hrr.										
	–Mar. 21,	10.00 „	24·17	295	2·340	0·097	65·00	2·70	2·78	165·0	6·8	210·0	8·6
2	Mar. 21,	10.00 a.m.											
	–Mar. 22,	10.15 „	24·25	300	2·448	0·101	49·80	2·05	2·02	155·0	6·4	209·0	8·6
3	–Mar. 22,	12.45 p.m.	2·50	29	0·281	0·112	4·97	1·99	1·77	18·0	7·2	12·8	5·1
4		–3.50 „	3·17	25	0·315	0·099	7·33	2·31	2·32	21·0	6·6	30·5	9·6
5		–6.45 „	2·83	45	0·279	0·099	7·07	2·50	2·54	19·5	6·9	28·1	9·9
6		–9.45 „	3·00	32	0·302	0·100	6·53	2·18	2·16	20·0	6·7	32·0	10·7
7	–Mar. 23,	9.45 a.m.	12·00	130	1·059	0·088	25·20	2·20	2·38	72·8	6·1	83·7	7·0
8	–Mar. 23,	12.45 p.m.	3·00	25	0·263	0·088	6·25	2·08	2·38	18·5	6·2	27·1	9·0
* 9		–3.45 „	3·00	—	0·330	0·110	5·80	1·93	1·76	21·0	7·0	5·9	1·9
10		–7.10 „	3·42	64	0·444	0·130	8·91	2·61	2·02	22·6	6·6	29·0	8·4
11		–10.00 „	2·83	60	0·204	0·070	3·96	1·40	1·94	15·5	5·5	60·0	21·2
12	–Mar. 24,	9.50 a.m.	11·83	120	0·884	0·075	19·65	1·66	2·22	67·9	5·7	100·1	11·6
13	–Mar. 24,	12.50 p.m.	3·00	35	0·244	0·081	5·40	1·80	2·21	21·1	7·0	12·9	4·3
14		–4.10 „	3·33	38	0·283	0·085	5·75	1·93	2·04	20·7	6·2	12·6	3·8
15		–6.50 „	2·67	25	0·189	0·071	2·84	1·06	1·50	16·7	6·3	4·8	1·8
16		–9.50 „	3·00	30	0·282	0·094	4·64	1·55	1·64	21·2	7·1	20·0	6·7
17	–Mar. 25,	9.50 a.m.	12·00	150	0·954	0·080	29·30	2·44	3·08	74·5	6·2	112·0	9·3
†18	–Mar. 25,	12.50 p.m.	3·00	45	—	—	—	—	—	(21·0)	—	—	—
19		–4.10 „	3·33	25	0·337	0·101	7·78	2·34	2·31	23·4	7·0	2·4	0·7
20		–6.55 „	2·75	70	0·467	0·170	10·37	3·79	2·22	16·7	6·1	0·6	0·2
21		–9.50 „	2·92	50	0·337	0·115	6·76	2·32	2·00	18·2	6·2	82·5	28·2
22	–Mar. 26,	9.55 a.m.	12·08	120	0·977	0·081	24·20	2·00	2·50	69·8	5·8	111·5	9·5
23	–Mar. 26,	12.55 p.m.	3·00	27	0·227	0·079	3·45	1·15	1·52	19·5	6·5	2·5	0·8
24		–3.50 „	2·92	29	0·232	0·080	5·60	1·92	2·41	18·6	6·4	14·4	4·9
25		–6.55 „	3·08	53	0·249	0·081	6·15	2·00	2·47	19·5	6·3	21·3	6·9
26		–10.00 „	3·08	38	0·262	0·085	7·08	2·30	2·70	20·0	6·5	30·6	9·9
27	–Mar. 27,	9.55 a.m.	11·92	140	0·910	0·076	25·60	2·15	2·82	72·8	6·1	83·5	7·0
‡28	–Mar. 27,	12.45 p.m.	2·83	20	0·220	0·078	4·85	1·71	2·20	17·6	6·2	1·6	0·6
§29		–3.45 „	3·00	80	0·366	0·122	7·27	2·42	1·98	17·7	5·9	trace	trace
‖30		–6.50 „	3·08	50	0·273	0·089	5·10	1·66	1·87	13·3	4·3	„	„
¶31		–9.50 „	3·00	25	0·278	0·093	9·36	3·12	3·37	18·5	6·2	75·0	25·0
32		–11.55 „	2·08	20	0·213	0·102	4·89	2·35	2·30	10·5	5·0	56·7	27·3
33	–Mar. 28,	9.55 a.m.	10·00	85	0·870	0·087	22·00	2·20	2·53	57·9	5·8	67·2	6·7
34	–Mar. 28,	12.50 p.m.	2·92	40	0·280	0·091	5·86	2·00	2·10	22·0	7·5	4·1	1·4
35		–3.50 „	3·00	47	0·263	0·088	7·26	2·42	2·76	18·0	6·0	15·0	5·0
36		–6.50 „	3·00	32	0·254	0·085	5·40	1·80	2·12	18·2	6·1	10·5	3·2
37		–9.50 „	3·00	30	0·248	0·083	6·56	2·19	2·65	17·9	6·0	23·2	7·8
38	–Mar. 29,	9.50 a.m.	12·00	140	0·988	0·082	23·70	1·98	2·40	61·7	5·1	100·1	8·4
39	–Mar. 29,	12.50 p.m.	3·00	30	0·249	0·083	5·34	1·78	2·15	18·2	6·1	6·9	2·3
40		–4.00 „	3·17	42	0·286	0·090	6·39	2·01	2·24	18·2	5·7	19·5	6·2
41		–6.45 „	2·75	36	0·218	0·079	3·08	1·12	1·41	15·6	5·7	8·0	3·0
42	–Mar. 30,	9.55 a.m.	15·17	170	1·120	0·074	21·55	1·42	1·92	83·8	5·5	127·2	8·4

* Insulin 6 units at 12.45 p.m. (weight 5·5 kg.).
† Insulin 9 units (weight 5·3 kg.).
‡ Insulin 9 units at 9.55 a.m. (weight 5·0 kg.).
§ Partial collapse from 12.45–3.0 p.m.
‖ Insulin 3 units at 3.45 p.m. Convulsions and coma at 6.45 p.m.
¶ Dog better at 9.45 p.m.

Dog F (continued)

No.	Period	Time	Duration	Urine cc.	Total N. g. Total	Total N. g. Per hour	Ammonia N. mg. Total	Ammonia N. mg. Per hour	Ammonia N. mg. Per cent.	Creatinine mg. Total	Creatinine mg. Per hour	Phosphorus mg. Total	Phosphorus mg. Per hour
			hrs.										
43	–Mar. 31,	9.55 a.m.	24·00	170	—	—	43·25	—	—	127·0	5·3	151·1	—
44	–Apr. 1,	9.55 a.m.	24·00	170	1·904	0·079	44·94	1·87	2·36	—	—	144·5	6·0
45	–Apr. 2,	9.50 a.m.	23·92	230	1·750	0·073	43·70	1·83	2·50	—	—	108·0	4·5
*46	–Apr. 2,	12.50 p.m.	3·00	40	0·242	0·081	5·93	1·97	2·45	—	—	20·9	7·0
47		–3.50 „	3·00	25	0·202	0·067	6·15	2·05	3·04	—	—	11·2	3·7
48		–6.50 „	3·00	35	0·206	0·069	6·77	2·26	3·29	—	—	trace	—
49		–9.55 „	3·08	115	0·207	0·069	—	—	—	—	—	12·2	4·0
50	-Apr. 3,	9.50 a.m.	11·92	155	0·479	0·040	18·08	1·51	3·77	—	—	100·0	8·4
51	–Apr. 3,	12.50 p.m.	3·00	14	0·095	0·032	3·02	1·01	3·18	—	—	4·9	1·6
52		–4.30 „	3·67	37	0·169	0·046	4·80	1·31	2·84	—	—	14·0	3·8
53		–6·50 „	2·33	28	0·102	0·043	3·09	1·33	3·03	—	—	5·0	2·1
54		–9.50 „	3·00	38	0·166	0·055	5·48	1·83	3·30	—	—	15·8	5·3
55	–Apr. 4,	9.50 a.m.	12·00	142	0·558	0·047	24·00	2·00	4·30	—	—	43·5	3·6
56	–Apr. 4,	12.55 p.m.	3·08	28	0·140	0·045	4·89	1·58	3·50	—	—	23·0	7·5
57	–Apr. 5,	9.50 a.m.	20·92	70	1·341	0·064	24·33	1·16	1·81	—	—	69·0	3·3
58	–Apr. 6,	9.50 a.m.	24·00	285	1·315	0·055	39·10	1·63	2·97	—	—	86·8	3·6
59	–Apr. 7,	9.50 a.m.	24·00	250	1·312	0·055	39·20	1·63	2·98	—	—	90·9	3·8
†60	–Apr. 7,	12.50 p.m.	3·00	28	0·209	0·070	5·57	1·86	2·66	—	—	11·3	3·8
61		–3.50 „	3·00	40	0·190	0·063	7·28	2·43	3·83	—	—	18·5	6·2
62		–7.00 „	3·17	70	0·210	0·066	6·15	1·94	2·93	—	—	4·9	1·5
63		–9.50 „	2·83	78	0·139	0·049	3·33	1·18	2·40	—	—	18·9	6·7
64	–Apr. 8,	9.50 a.m.	12·00	110	0·400	0·033	15·93	1·33	3·98	—	—	74·6	6·2
65	–Apr. 8,	12.50 p.m.	3·00	15	0·163	0·054	3·91	1·30	2·40	—	—	5·2	1·7
66		–3.55 „	3·08	35	0·138	0·045	3·05	0·99	2·21	—	—	4·9	1·6
67		–6.50 „	2·92	40	0·126	0·043	3·57	1·22	2·84	—	—	5·8	2·0
68		–9.55 „	3·08	40	0·136	0·044	5·16	1·68	3·80	—	—	19·2	6·2
69	–Apr. 9,	9.50 a.m.	11·92	145	0·504	0·042	16·51	1·39	3·28	—	—	33·5	2·8
70	–Apr. 10,	9.50 a.m.	24·00	325	1·119	0·047	43·30	1·80	3·88	—	—	6·9	2·9

* 25 g. sugar, insulin 10 units.
† 25 g. glucose.

daily rhythm in the excretion of phosphorus was noted on the normal days. Single doses of insulin were injected on two occasions, 6 units on March 23rd and 9 units on March 25th, but no symptoms developed on either date. The decrease and increase in phosphorus excretion occurred as before, the greater change following the larger dose. In the latter case the phosphorus almost disappeared from the urine. The increase for the day amounted to about 20 and 30 % respectively.

The nitrogen output was increased in the first 3 hours in each case, reaching the maximum in the second 3 hours, then falling. There was little if any increase for the whole day in the first case and a small increase, amounting to about 10 %, in the second case.

On March 27th, 9 units of insulin, given at 9.55 a.m., were followed by moderately severe symptoms—salivation, tremors, ataxia and partial collapse.

The animal recovered and a second dose of 3 units was administered at 3.45 p.m. Convulsions and coma occurred between 6 and 7 p.m., but the dog recovered without requiring sugar. The first injection was followed by almost complete disappearance of phosphorus from the urine and the second prolonged this condition for another three hours. Then the inevitable rise in phosphate occurred, probably lasting for several hours during the night. The total for the day was increased about 30 %.

The nitrogen rose immediately after the first injection, falling again after 6 hours. The second injection caused another increase, though smaller, reaching its peak in 6 hours. The total for the day was 2·220 g. and for the following day 2·033 g., compared with 1·880 g. the day before and 1·873 g. the second day after. The ammonia followed the total nitrogen excretion very closely in these experiments.

The creatinine excretion measured for the first 8 days showed very little change from day to day, or for the different periods during the day although there appeared to be a slightly higher output in the morning than in the afternoon and night. This difference, though apparently very constant, is probably not great enough to be of any significance. Administration of insulin did not affect the creatinine excretion on the days in which single doses were given. On March 27th, however, when the animal was in a state of collapse as the result of a second injection of insulin, the creatinine output was 4·3 mg., the normal being from 5·5 to 6·0 mg.

On April 7th, 25 g. of glucose (5 g. per kilo.) were given at 12.50 p.m. The phosphate excretion showed the usual changes but the effect was delayed, as it will be recalled was also the case when sugar was given to Dog *B* on March 6th. The usual afternoon rise continued in the first period after sugar was given but a decided fall took place in the next period, 3 to 6 hours later. In the subsequent period the increase had developed but the maximum was probably reached during the night. The total for the day was increased over 30 %.

The nitrogen output showed a gradual diminution, the lowest rate of excretion being reached during the night. The total for the day was decreased from 1·312 g. to 1·148 g., that of the day following being decreased still more—to 1·067 g.

On April 2nd, at 12.50 p.m., 25 g. of sugar and 10 units of insulin were given together. The phosphate excretion dropped at once, becoming a trace only in the second period. The increase which followed reached its maximum during the night. The total increase for the day amounted to over 40 %, and on the following morning the phosphate excretion was decidedly low. A further depression of the usual low morning phosphate seems to occur regularly on the days following large changes in phosphate excretion whether brought about by insulin, or sugar, or both together. This feature was very prominent on March 26th and 28th.

The volume of urine was diminished on March 25th in the first period after insulin from 15 to 7 cc. per hour. On March 27th, immediately after the first

dose of insulin, the excretion was low—8 cc. per hour—but in the next two periods, when the animal was in collapse, it was high, 26 and 16 cc. per hour.

II. *Diabetic animals.*

Depancreatised dogs were investigated in the same way as normal animals except that, as a rule, 100 cc. of water instead of 50 cc., were given before each day period, and 250 cc. at night.

Dog 23 had been depancreatised several months previously, being meanwhile fed on a diet of meat and cane sugar, with insulin twice daily most of the time. It was in good condition and had maintained its weight of about 7 kilos.

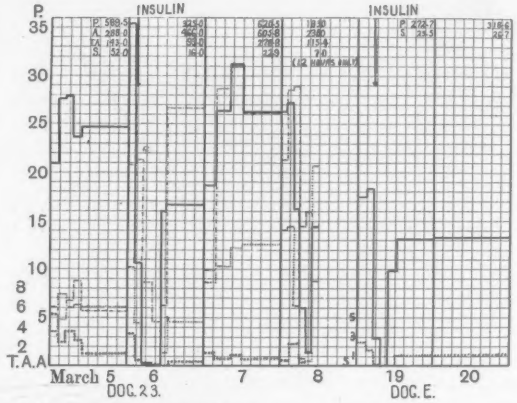

Fig. 4. Diabetic dogs 23 and *E*. Phosphorus ———. Sugar ∘ ∘ ∘ ∘ ∘. Ammonia – ∘ – ∘ – ∘ –. Titratable acidity .....

On March 5th, it was given no food or insulin and on this day the excretion of phosphate was extremely high, the total output for the day, nearly 600 mg., being more than double the output of a normal dog of similar weight. The high rate of excretion in the morning became still higher in the afternoon, this change being similar to the normal diurnal variation. The urinary sugar was high in the morning but fell during the day, probably on account of loss of glycogen deposited as a result of the sugar feeding and insulin treatment on the preceding days.

On March 6th insulin (15 units) was given at 12.10 p.m. The phosphate excretion, which had been extremely high in the morning (35·30 mg. per hour) fell immediately, almost reaching zero in the period from 5 to 7 hours after insulin. This was followed by an increase to 16·78 mg. per hour during the night. The total for the day was 333·86 mg., which is about 50 % less than the excretion on the preceding and following days. The sugar and acetone bodies disappeared immediately after the injection, returning in about 8 hours.

On March 8th, a dose of 8 units of insulin was given with similar results. The fall in phosphate was not quite as great, though a decrease from 26 mg.

per hour to 1·41 mg. took place in 7 hours. An increase to 14·42 mg. followed. The experiment had to be terminated, on account of symptoms, at 9.30 p.m., before the phosphate had risen to its highest level.

The excretion of ammonia paralleled fairly closely that of the phosphate. Changes in the titratable acidity corresponded almost exactly with the changes in phosphate. When the phosphate was low after insulin the urine became almost neutral on both occasions.

Dog D, also depancreatised several months previously, had been fed meat and sugar and received insulin before each feeding. On March 11th, the day before observations were begun, an overdose of insulin was injected and severe hypoglycaemic symptoms developed. Administration of glucose was necessary to restore the animal.

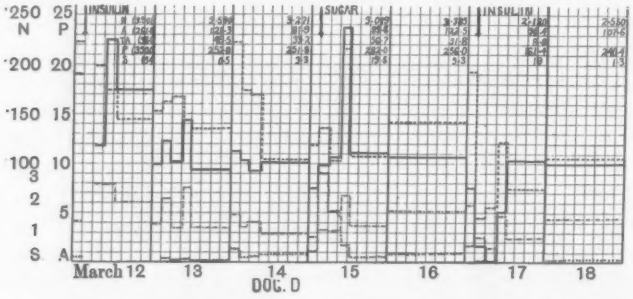

Fig. 5. Diabetic dog *D*. Phosphorus ———. Sugar ∘ ∘ ∘ ∘ ∘. Ammonia –∘–∘–∘–. Nitrogen – – – – –.

On March 12th the dog was apparently in good condition, its weight being 7·0 kilos. It was given 15 units of insulin at 1 p.m. and again hypoglycaemic symptoms with mild convulsions developed. Temporary recovery took place after giving about 7 g. of cane sugar by stomach tube at 4 p.m. At 7 p.m. the dog was found to be in a comatose state with slow irregular heart and respiration. It was given 10 g. of glucose subcutaneously. It recovered consciousness but was still very weak at 10.20 p.m.

The phosphate excretion before insulin was given amounted to 19 mg. per hour which is two or three times the normal value. After insulin a decrease occurred but in the 3rd period (9 to 12 hours) the excretion (22·6 mg. per hour) had returned to the original level.

The excretion of nitrogen showed a steady decrease, reaching the lowest level during the night.

On March 13th and 14th the dog appeared normal; no food or insulin was given though the animal seemed to be still under the influence of insulin to some extent, since the amount of sugar excreted was small and acetone bodies were absent from the urine.

On March 15th, 20 g. of glucose were given by stomach tube; most of it,

but not all, was excreted within the next 7½ hours, the total output for this period being 15·5 g. The phosphate excretion showed a marked increase in the 3rd period, 7 hours after the glucose, the nitrogen and ammonia being also increased in this period.

During the night the excretion of all three had again returned to normal. The usual preliminary decrease found after giving sugar to normal animals was not observed, perhaps because it was too small and of too short duration to be apparent in the periods over which urine was collected. Otherwise the result resembles that produced by giving sugar to a normal animal and, as more than 25 % of the sugar given was retained, may differ from the result of sugar ingestion on a diabetic where none is utilised.

On March 17th a smaller dose of insulin, 10 units, was given and no symptoms resulted. The phosphate fell immediately, becoming only a trace in the urine for the 2nd period. The increase which followed was not great and the original level was reached in the night.

The nitrogen diminished immediately, the decrease lasting for 6 hours, but a small increase took place during the night.

Dog E was depancreatised on February 14th. It was fed 200 g. meat and 50 g. sugar twice daily and received insulin before each feeding until March 19th, when no food was given. (Its weight was 7·5 kilos.) The phosphate excretion in the morning was high, as in the other diabetic dogs (17·30 mg. per hour rising to 18·18 mg. between 1 and 3 p.m.). 10 units of insulin were given at 3 p.m. with the result that there was an immediate decrease in phosphates followed after 5 hours by an increase which, however, did not bring the excretion to its original level. The average for the night was 13·10 mg. per hour.

Summary.

1. *Effect of Insulin on Normal Animals.* The administration of insulin to normal animals resulted in an immediate reduction in the excretion of inorganic phosphate in the urine. After a large dose, not sufficient, however, to cause symptoms of hypoglycaemia, the amount of phosphate became too small to measure. The period of reduced excretion lasted for about 6 hours, and was followed by a period of increased excretion, the maximum being reached in from 9 to 12 hours after the injection. The total output of phosphorus for the day was usually increased from 25 to 35 %, though in some cases the increase in phosphate excretion occurred partly or mainly on the following day.

The effect of repeating the dose was to prolong for a time the phase of diminished excretion. This was, however, followed by the inevitable increase. The cause of this cannot be that there is lack of insulin, or that the effect of insulin has passed off, since it was observed when the animals were obviously under the influence of insulin; for example, the increase occurred in Dog *F* when severe hypoglycaemic symptoms were still present. On the other hand, when Dog *A* was given three injections of insulin in one day the increase did

not balance the decrease which had lasted for about 9 hours, so that the total output for the day was diminished instead of being increased.

The nitrogen excretion showed an increase which was evident in the first 6 hours after insulin. On the average the total output for the day was increased 20 % and on March 20th, when Dog *A* was given three injections of insulin, the nitrogen output was approximately doubled.

The excretion of ammonia and the titratable acidity of the urine paralleled, in general, the excretion of phosphorus. On some occasions, however, the ammonia became greater while the phosphorus was low, but this increase corresponded with an increase in the total nitrogen output. The titratable acidity was always low after insulin, when the phosphate output was low, and on some occasions it became zero.

The volume of urine was usually diminished in the first 3- or 6-hour periods after insulin. The initial decrease was followed by a compensatory increase, usually appearing in the post-hypoglycaemic period. The increase occurred, however, in the case of Dog *F*, on March 27th, when severe hypoglycaemic symptoms were still present.

The excretion of creatinine does not appear to be affected by administration of insulin. It did not increase when there was an increased output of the total nitrogen.

2. *Effect of Sugar.* The ingestion of sugar caused changes in excretion similar to those caused by insulin. The decrease in phosphate was almost as great, but its onset appeared to be slower. On two occasions the usual afternoon increase continued for the 1st period after sugar ingestion; it always decreased during the 2nd period however. This was followed after 6 hours by a large increase but in only one case out of three was the phosphorus output for the 24 hours greater than normal.

The excretion of nitrogen gradually declined so that the total for the day was diminished as well as that for the following day. The ammonia fell in proportion to the fall in total nitrogen. The titratable acidity paralleled the excretion of phosphate.

3. *Effect of Insulin and Sugar.* Insulin and glucose given at the same time brought about the same changes in phosphate excretion as were observed after giving insulin or glucose separately. The nitrogen (Dog *F*, April 2nd) gradually diminished, reaching the lowest level during the night. The output for the day was decreased, but the greatest decrease was shown on the following day.

4. *Effect of Acid.* The administration of acid did not alter the excretion of phosphate.

5. *Effect of Insulin and Sugar on Diabetic Dogs.* The phosphorus excretion of diabetic dogs without insulin was very high, being more than twice that of the normal. Administration of insulin caused a decrease in the excretion, then an increase, with return to the level for a normal dog. The output for the 24 hours was reduced to a value approximating to the normal.

The one experiment in which sugar was given did not yield a clear cut result. Further experiments now in progress suggest that sugar will not affect the phosphate excretion if there is complete absence of insulin.

Discussion.

The striking changes in the excretion of phosphate following the administration of insulin together with the fact that the same changes occur after the ingestion of sugar establish beyond question the existence of an intimate relationship between the metabolism of carbohydrate and phosphoric acid. It would appear that after the ingestion of carbohydrate, or after administration of insulin when change in glucose is taking place, there is a demand for phosphate to form some compound with a carbohydrate product. This compound exists only temporarily and when it breaks up, the phosphoric acid is released, and the excretion therefore increased.

The rôle of phosphate in the fermentation of glucose demonstrated by Harden and Young and by Lebedev [see Harden, 1923], and the relationship of lactacidogen to muscular contraction established by Embden and others [1914], suggest that the hypothetical compound of phosphorus is hexosephosphate. Study of the lactacidogen and inorganic phosphate content of rabbit's muscle after insulin administration and after insulin along with sugar, made in this laboratory, has not, however, yielded results which could be considered to afford direct proof for this hypothesis. This is contrary to the results reported by Audova and Wagner [1924], and Harrop and Benedict who claim to have found an increase in lactacidogen in muscle after insulin. The technical difficulties of these experiments are very great, so that further work will be necessary to settle this point.

The increase in nitrogen excretion when insulin is given to a normal fasting animal, may indicate that when there is not sufficient carbohydrate present for the insulin to act upon, there is increased catabolism of protein to provide the necessary sugar. When, however, there is sufficient sugar available, as is the case in a normal well-fed animal, or in a fasting animal when sugar is given at the same time as insulin, there is a decrease in nitrogen excretion. Here the increased carbohydrate combustion exerts a protein-sparing action. It may be noted that previous work [Macleod and Allan, 1923] showed that when insulin was given to a well-fed animal, the nitrogen excretion was diminished, the greatest decrease frequently occurring on the day following the insulin administration. This result has been confirmed in the present observations when insulin and sugar were given together. The fact that the administration of insulin diminished the nitrogen loss of a fasting diabetic probably indicates not only that there is abundant sugar present in the body for it to act upon, but also that the previous rapid breakdown of protein giving rise to sugar, is retarded. The other explanation of protein-sparing action, namely that there is re-synthesis of amino acids from decomposition products of protein and carbohydrate metabolism must also be kept in mind.

The increase in the phosphorus output for the 24 hours did not occur except where there was also an increased nitrogen output (with one exception). In view of the continuous high phosphorus excretion in diabetic animals in which the nitrogen excretion is also high this observation suggests a common origin of the phosphorus and nitrogen. It may be that substances of the nature of nucleoprotein or inosinic acid furnish the material from which the sugar and phosphorus are derived. Another possibility which must be considered is that insulin is concerned with fat synthesis and that the phosphorus becomes incorporated in a phospholipin. These suggestions are under investigation.

The possibility that the changes in phosphate excretion are the result of alteration in the acid-base equilibrium can be dismissed. In an investigation of the effects of acidosis in cats (Sokhey, unpublished work) no change in urinary phosphate was found to occur, an observation which is confirmed in the present investigation. There is, however, a change in the reaction of the urine after insulin dependent no doubt on the change in phosphates. The parallelism between the titratable acidity of the urine and the phosphate content has shown itself to be remarkably close.

Conclusion.

The changes in excretion of inorganic phosphate in the urine, after ingestion of sugar and administration of insulin to normal and diabetic animals, are described in detail and the time relationships studied. The intimate association of the metabolism of carbohydrate and phosphoric acid is demonstrated. Coincident metabolic changes are discussed and their significance suggested.

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REFERENCES.

Audova and Wagner (1924). *Klin. Wochensch.* Feb. 5, No. 6.
Blatherwick, Bell and Hill (1923). *Proc. Amer. Soc. Biol. Chem.* **6**, 35.
Campbell and Webster (1921). *Biochem. J.* **15**, 660.
Elias and Weiss (1922). *Wien. Arch. inn. Med.* **4**, 29.
Embden, Griesbach and Laquer (1914). *Z. physiol. Chem.* **93**, 1.
Fiske (1920). *J. Biol. Chem.* **41**, lix.
—— (1921). *J. Biol. Chem.* **49**, 171.
Harden (1923). Alcoholic Fermentation. (Longmans, Green & Co.)
Harrop and Benedict (1923). *Proc. Soc. Exp. Biol. Med.* **29**, 430.
—— —— (1924). *J. Biol. Chem.* **59**, 683.
Macleod and Allan (1923). *Trans. Roy. Soc. Canada*, Section v, 47.
Perlyzweig, Lathan and Keefer (1923). *Proc. Soc. Exp. Biol. Med.* **21**, 33.
Wigglesworth, Woodrow, Smith and Winter (1923). *J. Physiol.* **57**, 447.
Winter and Smith (1924). *J. Physiol.* **58**, 327.